PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
A01N 43/04, A61K 31/70, C12N 15/00, 15/63, 15/70 — A1

(11) International Publication Number: WO 98/00014

(43) International Publication Date: 8 January 1998 (08.01.98)

(21) International Application Number: PCT/US97/11602

(22) International Filing Date: 27 June 1997 (27.06.97)

(30) Priority Data:
08/674,231 28 June 1996 (28.06.96) US

(71) Applicant *(for all designated States except US)*: THE REGENTS OF THE UNIVERSITY OF CALIFORNIA [US/US]; 22nd floor, 300 Lakeside Drive, Oakland, CA 94612 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: LALWANI, Anil [US/US]; 251 Molino Avenue, Mill Valley, CA 94941 (US). SCHINDLER, Robert, A. [US/US]; 32 States Street, San Francisco, CA 94114 (US).

(74) Agents: BOZICEVIC, Karl et al.; Fish & Richardson P.C., Suite 100, 2200 Sand Hill Road, Menlo Park, CA 94025 (US).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: TRANSFORMATION AND GENE THERAPY OF CELLS OF THE INNER EAR

(57) Abstract

The present invention features compositions and methods for transformation of cells of the inner ear and treatment of conditions of the inner ear using such methods. More specifically, cells of an inner ear of a subject are genetically altered to operatively incorporate a nucleotide sequence which expresses a gene product of interest (e.g., a therapeutic gene product). Preferably, the inner ear cell into which the DNA of interest is introduced and expressed is a cell of the cochlea, more preferably a cell of the spiral ligament, spiral limbus, stria vascularis, organ of Corti, spiral ganglion, and/or Reissner's membrane, and/or an auditory hair cell. The DNA of interest, preferably present within an adeno-associated viral vector, is introduced through a cannula inserted in the round or oval window and in communication with the perilymph or endolymph. Preferably, introduction of the DNA of interest is accomplished using an osmotic minipump.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

TRANSFORMATION AND GENE THERAPY OF CELLS OF THE INNER EAR

Field of the Invention

This invention relates generally to the field of eukaryotic cell transformation, particularly transformation of cells of the inner ear, including cells of the auditory nerves, cochlea, and vestibular system, and application of transformation techniques of the invention for use in gene therapy.

Background of the Invention

The ear facilitates sound perception by first transforming the pressure changes of sound waves into physical movements of the tympanic membrane (Figs. 1 and 2). Movements of the tympanic membrane are then translated into movements of the bones of the middle ear, so that the footplate of the stapes induces movements of the oval window. These oval window movements are transmitted as waves in the endocochlear fluids (perilymph, endolymph, and cortilymph). Pressure waves induced in the cochlear fluids then cause movements of the basilar membrane of the organ of Corti, located in the cochlear duct (Fig. 3), thereby inducing a shearing or twisting motion in the process of the hair cells whose distal tips are embedded in the tectorial membrane. The hair cells transduce the mechanical deformation of the hairs into nerve impulses in the terminal fibers of the cochlear branch of the vestibulocochlear nerve (cranial nerve VIII). Transmission of these signals to the appropriate cortical areas of the brain results in sound perception.

Hearing impairment, which can range from a minor inability to perceive particular sound frequencies to complete deafness, is classified into three general categories: conductive losses, sensorineural losses, and mixed losses. Conductive losses interfere with transmission of sound to the cochlea, and are usually caused by abnormalities of the outer and middle ear. For example, total occlusion of the external canal by cerumen is the most common cause of a conductive

hearing loss in elderly patients, while fluid in the middle ear space (serus otitis media) is the most common cause of conductive hearing loss in children.

Sensorineural hearing loss is associated with damage to the cochlea, cranial nerve VIII, or its central connection (the brain stem auditory pathways and the auditory crest). Damage can result from age-related changes, environmental influence, or pathological processes. For example, presbycusis, a sensorineural hearing loss that accompanies aging, is caused by a degenerative process of the inner ear and is characterized by a loss of hair cells, atrophy of the spinal ganglion, altered endolymph production, thickening of the basilar membrane, or neural degeneration. Other causes of sensorineural losses include noise trauma, ototoxic drugs, involvement of the acoustic nerve by benign or malignant tumors, cerebrovascular disease, and possible complications of arteriosclerosis. Hearing loss that is caused by both conductive and sensorineural impairments is termed mixed or combined hearing loss.

Conventional treatment of hearing loss depends on the type and degree of hearing loss and the age at onset. Surgical and medical intervention, corrective amplification (e.g., hearing aids), and education are most commonly used. Medical management, surgical management, or both are generally appropriate for conductive hearing loss, depending on the degree of impairment and the status of bone conduction and speech discrimination. Surgical removal of tumors of cranial nerve VIII may preserve hearing; however, the primary objective of such procedures is relief of vertigo. Cochlear lesions usually are not correctable using conventional therapies. Management of a disease or damaged cochlea using conventional therapy consists of hearing aid fitting, audiological rehabilitation, and for patients without aidable hearing, cochlear implants.

The conventional methods of treatment and study of hearing loss associated with the inner ear are limited in scope and success. Furthermore, because the structures of the inner ear are so delicate, attempts to study the mechanisms of hearing and hearing loss are severely hampered by the lack of

adequate means to examine the function of specific genes and gene products within the cells of the inner ear. Although gene therapy is currently used to treat a variety of genetic conditions and infections in other settings, application of gene therapy to genetic defects and other conditions of the inner ear is hampered by the availability of a method for delivery of transforming DNA to the inner ear and transformation of the cells.

The present invention addresses this problem.

Summary of the Invention

The present invention features compositions and methods for transformation of cells of the inner ear and treatment of conditions of the inner ear using such methods. More specifically, cells of an inner ear of a subject are genetically altered to operatively incorporate a nucleotide sequence which expresses a gene product of interest (e.g., a therapeutic gene product). Preferably, the inner ear cell into which the DNA of interest is introduced and expressed in a cell of the cochlea, more preferably a cell of the spiral ligament, spiral limbus, stria vascularis, organ of Corti, spiral ganglion, and/or Reissner's membrane, and/or an auditory hair cell. The DNA of interest, preferably present within an adeno-associated viral vector, is introduced through a cannula inserted in the round or oval window and in communication with the perilymph or endolymph. Preferably, introduction of the DNA of interest is accomplished with an osmotic minipump.

The invention also features recombinant cells of the inner ear, preferably a recombinant cochlear cell, more preferably a recombinant cell of the spiral ligament, spiral limbus, organ of Corti, spiral ganglion, Hansen's cell, and/or Reissner's membrane and/or an auditory hair cell, containing a DNA of interest operatively inserted in the genome of the cell and operatively linked to a promoter for expression of the DNA of interest. In a related embodiment, the invention features an isolated cochlea (e.g, in an organotypic cochlear culture) which cochlea contains transformed cochlear cells.

A primary object of the invention is to provide a method for transformation of inner ear cells useful in the examination of the mechanisms associated with hearing and hearing impairment.

Another object is to provide a method of gene therapy wherein cells of an inner ear of a subject are genetically modified to express a therapeutically useful gene product.

Another object is to produce genetically transformed inner ear cells which cells have incorporated into their genome genetic material which expresses a gene product of interest, e.g., a therapeutic gene product.

An advantage of the present invention is that gene therapy can be achieved in the ear to provide treatment of various conditions associated with defects (e.g., genetic defects, or defects associated with environmental damage or age-related deterioration) and/or infections of the peripheral auditory system, including the cochlea and its associated cells and structures.

Another advantage is that the method of transformation of the invention provides a means to access the inner ear and transform cells of the inner ear without substantially disrupting the delicate structures of the inner ear or eliciting a significant inflammatory response.

Yet another advantage is that the method of transformation of the invention can be used in conjunction with cochlear implantation to deliver various factors to enhance acceptance and success of the implant (e.g., antiinflammatory agents, nerve growth factors, antibiotic agents, etc.).

Another advantage of the present invention is that transformation of the cells of the peripheral auditory system, including cells of the cochlea, can be used to examine the causes of hearing impairment and to better understand the mechanisms associated with hearing and balance.

These and other objects, advantages and features of the present invention will become apparent to those persons skilled in the art upon reading the details of the vectors, cell lines and methodology as more fully set forth below.

Brief Description of the Drawing

Fig. 1 is a schematic illustration of the human ear.

Fig. 2 is a schematic illustration of the human inner ear.

Fig. 3 is a schematic illustration of a cross-section through a turn of a human cochlea.

Fig. 4 is a schematic illustration (enlarged relative to Fig. 3) of a cross-section through a turn of a human cochlea.

Fig. 5 is a schematic of a recombinant construct useful in producing recombinant inner ear cells according to the invention.

Fig. 6 is a schematic view of a recombinant adeno-associated viral construct containing DNA encoding the marker gene β-galactosidase (β-gal). TR represents the terminal repeat of adeno-associated virus (145 bp); P_{MAP} represents the Ad 2 major late promoter (695 bp); SD/SA SV40 represents the late gene 16S/19S splice donor/splice acceptor signal of SV40 (180 bp); β-gal represents that β-gal gene (3530 bp); pAl represents the polyadenylation signal of the SV40 late genes (196 bp); and pAe represents the polyadenylation signal of SV40 early genes (50 bp).

Fig. 7 is a schematic illustration of a recombinant adeno-associated viral vector for expression of neurotrophin-3 (NT-3).

Fig. 8 is a schematic illustration of a recombinant adeno-associated viral vector for expression of brain-derived neurotrophin factor (BDNF).

Fig. 9 is a schematic illustration of a recombinant adeno-associated viral vector for expression of nerve growth factor (NGF).

Fig. 10 is a schematic illustration of a recombinant adeno-associated viral vector expressing β-galactoidase under the control of the CMV immediate/early gene promoter (Pcmv). IRES represents the internal ribosome entry site; SV40 SD/SA represents the late gene 16S/19S splice donor/splice acceptor signal of SV40; SV40poly(A) represents polyadenylation signal of SV40 late genes; bGHpoy(A) represents the polyadenylation signal.

Fig. 11 is a schematic illustration of a recombinant adeno-associated viral vector for expression of green fluorescent protein (GFP) from the CMV early/immediate gene promoter(Pcmv). IRES represents the internal ribosome entry site; SV40 SD/SA represents the late gene 16S/19S splice donor/splice acceptor signal of SV40; SV40poly(A) represents polyadenylation signal of SV40 late genes; bGHpoy(A) represents the polyadenylation signal.

Fig. 12 is a schematic illustrating use of the osmotic minipump for steady state delivery of AAV to the cochlea of a guinea pig via a cannula inserted through an opening created near the cochlear base.

Fig. 13 is a panel of photographs showing *in vitro* transfection of rat cochlear cells either as dissociated cells (upper left panel) or as cochlear explants (bottom left and right panels).

Fig. 14 is a panel of photographs showing organotypic rat cochlear explants stained with neurofilament antibody after exposure to amikacin either with or without transformation with BDNF-encoding DNA (AAV-BDNF). Control, untreated cells, top left panel; cells treated with amikacin only, top right panel; cells infected with AAV-BDNF, bottom left and right panels.

Description of the Preferred Embodiments

Before the present method of genetically transforming cochlear cells and methods for providing gene therapy are described, it is to be understood that this invention is not limited to the particular methodology, protocols, cell lines, cochlear cells, vectors and reagents described as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention which will be limited only by the appended claims.

It must be noted that as used herein and in the appended claims, the singular forms "a", "and", and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a cochlear cell" includes a plurality of such cells and reference to "the

transformation vector" includes reference to one or more transformation vectors and equivalents thereof known to those skilled in the art, and so forth.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which this invention belongs. Although any methods, devices and materials similar or equivalent to those described herein can be used in the practice or testing of the invention, the preferred methods, devices and materials are now described.

All publications mentioned herein are incorporated herein by reference for the purpose of describing and disclosing the cell lines, vectors, and methodologies which are described in the publications which might be used in connection with the presently described invention. The publications discussed above and throughout the text are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the inventors are not entitled to antedate such disclosure by virtue of prior invention.

Definitions

By "inner ear" is meant the portion of the ear positioned medial to the middle ear of a mammalian or nonmammalian subject, and includes the cochlea, the auditory nerves, the semicircular canals and ducts, the ampules, the saccule, the endolymphatic sac, and the cochlear and vestibular aqueducts. Fig. 1 is a schematic showing the position of the inner ear relative to the outer and middle ear. Fig. 2 is a schematic showing a cross section of the inner ear and its relation to the middle ear.

By "inner ear cell" or "cell of the inner ear" is meant a cell of any of the structures of the inner ear.

By "cochlea" is meant the structure of the inner ear in the shape of a coiled tube of about 35 mm in length. In humans the cochlea makes about 2 3/4 turns. The cochlea is divided into three chambers, or scala, throughout its length by the basilar membrane and Reissner's membrane. The upper scala vestibuli and the lower scala tympani contain perilymph

and communicate with each other at the apex of the cochlea through a small opening called the helicotrema. At the base of the cochlea, the scala vestibuli ends at the oval window, which is closed by the foot plate of the stapes. The scala tympani ends at the round window, a foramen on the medial wall of the middle ear that is closed by the flexible secondary tympanic membrane. The scala media, the middle cochlear chamber that contains endolymph, is continuous with the membranous labyrinth and does not communicate with the other two scalae. Figs. 2 and 3 are schematics of a the human cochlea and structures within the human cochlea. Fig. 4 is a schematic of a cross section of a turn of the cochlea of the guinea pig.

By "isolated cochlea," "cultured cochlea," or "organotypic cochlear culture" is meant a cochlea that is removed and separate from an inner ear.

By "cochlear cell" is meant a cell of any portion of or structure of the cochlea, including, but not limited to, any cell of the spiral ligament, spiral limbus, stria vascularis, organ of Corti, Reissner's membrane, basilar membrane, and spiral ganglion, as well as auditory hair cells. Structures and cells of the cochlea are shown in Figs. 3 and 4. A substantially enriched transformed cochlear cell culture is an *in vitro* culture of cochlear cells genetically transformed according to the method of the invention.

"Window of the inner ear" is meant to include the round window and oval window of the inner ear. The round window is a foramen on the medial wall of the middle ear positioned at the at the base of the cochlea at the end of the scala tympani. The round window is closed by the secondary tympanic membrane. The oval window is a foramen that defines the end of the scala vestibuli and is closed by the foot plate of the stapes. The position of the oval and round windows in the ear are shown in Fig. 2.

By "transformation" is meant any permanent or transient genetic change induced in a cell following incorporation of new DNA (i.e., DNA exogenous to the cell). The new DNA can be present in the cell as an extrachromosomal or chromosomally integrated element.

By "target cell" is meant a cell(s) that is to be transformed using the methods and compositions of the invention. Transformation may be designed to nonselectively or selectively transform the target cell(s). In general, target cell as used herein means a cell of the inner ear that is to be transformed using the method and compositions of the invention.

By "transformed cell" is meant a cell into which (or into an ancestor of which) has been introduced, by means of recombinant DNA techniques, a DNA molecule encoding a molecule (e.g., RNA and/or protein) of interest (e.g., nucleic acid encoding a therapeutic cellular product).

By "nucleotide sequence of interest" or "DNA of interest" is meant any nucleotide sequence (e.g., RNA or DNA sequence) or DNA sequence that encodes a protein or other molecule that is desirable for expression in a target cell (e.g., for production of the protein or other biological molecule (e.g., a therapeutic cellular product) in the target cell). The nucleotide sequence of interest is generally operatively linked to other sequences which are needed for its expression, e.g., a promoter. Use of "DNA of interest" throughout the specification is not meant to limit the invention to deoxyribonucleic acid.

By "gene product of interest" is meant a polypeptide, RNA molecule, or other gene product that is desired for expression in a cell of the inner ear. "Gene products of interest" can include, for example, polypeptides that serve as marker proteins to assess cell transformation and expression, fusion proteins, polypeptides having a desired biological activity, gene products that can complement a genetic defect, RNA molecules, transcription factors, and other gene products that are of interest in the regulation and/or expression of the cellular functions of inner ear cells. "Gene products of interest" can also include nucleotide sequences that provide a desired effect or regulatory function, but do not necessarily encode an RNA molecule or polypeptide per se (e.g., transposons, introns, promoters, enhancers, splice signals, etc.).

By "therapeutic gene product" is meant a polypeptide, RNA molecule or other gene product that, when expressed in a cell of the inner ear, provides a desired therapeutic effect, e.g., repair of a genetic defect in the inner ear cell genome (e.g., by complement), expression of a polypeptide having a desired biological activity, and/or expression of an RNA molecule for antisense therapy (e.g., regulation of expression of a endogenous or heterologous gene in the inner ear cell genome).

By "vector" is meant any compound or formulation, biological or chemical, that facilitates transformation or transfection of a target cell (e.g., a cochlear or vestibular cell) with a DNA of interest. Exemplary biological vectors include viruses, particularly attenuated and/or replication-deficient viruses. Exemplary chemical vectors include lipid complexes and DNA constructs.

By "promoter" is meant a minimal DNA sequence sufficient to direct transcription of a DNA sequence to which it is operably linked. "Promoter" is also meant to encompass those promoter elements sufficient for promoter-dependent gene expression controllable for cell-type specific expression, tissue-specific expression, or inducible by external signals or agents; such elements may be located in the 5' or 3' regions of the native gene.

By "operably linked" is meant that a DNA sequence and a regulatory sequence(s) are connected in such a way as to permit gene expression when the appropriate molecules (e.g., transcriptional activator proteins) are bound to the regulatory sequence(s).

By "operatively inserted" is meant that the DNA of interest is positioned adjacent a DNA sequence that directs transcription and translation of the introduced DNA (i.e., facilitates the production of, e.g., a polypeptide encoded by a DNA of interest).

By "subject" or "patient" is meant any mammalian or nonmammalian subject for which inner ear cell transformation and/or gene therapy is desired. Such subjects include, but are not limited to, humans, guinea pigs, primates, mice, cattle, goats, sheep, horses, dogs, cats, chicks, chick

embryos, bullfrogs (as well as other reptiles), and fish. Subjects include subjects that are to be treated for a hearing disorder or condition (e.g., humans) as well as mature and immature subjects that can serve as models for the study of hearing, cochlear and vestibular function, hearing loss, disorders, and conditions associated with hearing loss.

By "transgenic organism" is meant a nonhuman organism (e.g., mammal or nonmammal), having a nonendogenous (i.e., heterologous) nucleic acid sequence present as an extrachromosomal element in a portion of its cells or stably integrated into its germ line DNA.

By "transgenic animal" is meant a nonhuman animal subject, usually a mammal, having a nonendogenous (i.e., heterologous) nucleic acid sequence present as an extrachromosomal element in a portion of its cells or stably integrated into its germ line DNA (i.e., in the genomic sequence of most or all of its cells). Heterologous nucleic acid is introduced into the germ line of such transgenic animals by genetic manipulation of, for example, embryos or embryonic stem cells of the host animal.

The invention will now be described in further detail.

Vectors and constructs

Any of a variety of vectors may be used in the present invention. Exemplary biological vectors include viruses, particularly attenuated and/or replication-deficient viruses. Exemplary chemical vectors include lipid complexes and various formulations comprising the nucleotide sequence of interest. The vectors can contain or be derived from any of a variety of viral constructs, bacterial constructs, or constructs capable of replication in eukaryotic and prokaryotic hosts. Preferably, the construct is capable of replication in both eukaryotic and prokaryotic hosts in order to facilitate efficient production of the DNA of interest for use in the method of the invention. Numerous constructs that can replicate in eukaryotic and prokaryotic hosts are known in the art and are commercially available. The construct may be a stably integrating construct or a stable nonintegrating construct. Examples of such constructs include viral

constructs and artificial chromosomes (e.g., human artificial chromosomes). A generic construct for use in the method of the invention is shown in Fig. 5. The basic vector components include a promoter operably linked to a nucleotide sequence of interest. Additional components of a basic vector include a polyadenylation signal, a splice signal, and terminal repeat sequences (TR), e.g., TR sequences corresponding to the viral sequence from which a viral vector is derived.

Transformation of inner ear cells may be accomplished by introduction of a DNA- or RNA-liposome complex formulations into the inner ear. DNA- or RNA- complex formations comprise a mixture of lipids which bind to genetic material (DNA or RNA), providing a hydrophobic coat which allows the genetic material to be delivered into cells. Liposomes which can be used in accordance with the invention include DOPE (dioleyl phosphatidyl ethanol amine), CUDMEDA (N-(5-cholestrum-3-β-ol 3-urethanyl)-N',N'-dimethylethylene diamine). When the DNA of interest is introduced using a liposome, it is preferable to first determine *in vitro* the optimal values for the DNA:lipid ratios and the absolute concentrations of DNA and lipid as a function of cell death and transformation efficiency. These values can then be used in or extrapolated for use in *in vivo* transformation. The *in vitro* determinations of these values can be readily carried out using techniques which are well known in the art.

Other nonviral vectors may also be used in accordance with the present invention. For example, such chemical formulations include DNA or RNA coupled to a carrier molecule (e.g., an antibody or a receptor ligand) which facilitates delivery to host cells for the purpose of altering the biological properties of the host cells. By the term "chemical formulations" is meant modifications of nucleic acids to allow coupling of the nucleic acid compounds to a carrier molecule such as a protein or lipid, or derivative thereof. Exemplary protein carrier molecules include antibodies specific to the cells of a targeted inner ear cell or receptor ligands, i.e., molecules capable of interacting with receptors associated with a cell of a targeted inner ear cell. Alternatively, the DNA of interest may be naked (i.e.,

not encapsulated), or may be provided as a formulation of DNA and cationic compounds (e.g., dextran sulfate, DEAC-dextran, or poly-L-lysine).

Preferably, a viral vector is used in the method of inner ear gene therapy of the invention. In general, viral vectors used in accordance with the invention are composed of a viral particle derived from a naturally-occurring virus which has been genetically altered to render the virus replication-defective and to express a recombinant gene of interest. Once the virus delivers its genetic material to a cell, it does not generate additional infectious virus but does introduce exogenous recombinant genes into the cell, preferably into the genome of the cell. Alternatively, the virus containing the DNA of interest is attenuated, i.e. does not cause significant pathology or morbidity in the infected host (i.e., the virus is nonpathogenic or causes only minor disease symptoms). Numerous viral vectors are well known in the art, including, for example, adeno-associated virus (AAV), retrovirus, adenovirus, herpes simplex virus (HSV), cytomegalovirus (CMV), vaccinia and poliovirus vectors. In addition, lentivirus may be used to deliver a DNA of interest to inner ear cells.

Several viral vectors have designed for delivery of nucleotide sequences encoding therapeutic gene products to eukaryotic cells (Cohen-Haguenauer, 1994, *Nouvelle Revue Francaise D Hematologie*, 36 Suppl 1:S3-9). The prototypes for viral mediated gene transfer are the retroviruses (Williams, 1990, *Hum. Gene Therap.*, 1(3):229-39; Merrouche et al., 1992, *Hum. Gene Therap.*, 3(3):285-91; Barba et al, 1993, *J. Neurosurg.*, 79(5):729-35). Retroviral vectors are characterized by their ability to preferentially integrate into the genome of rapidly dividing cells, making them an ideal vector for introducing tumoricidal factors into proliferating neoplastic cells. However, retroviral vectors are not necessarily as well-suited to the transformation of the neurosensory epithelia of the inner ear, which are post-mitotic and thus not rapidly dividing. Adenoviral vectors infect both dividing and nondividing cells with high efficiency. Adenoviral vectors do not integrate into the

genome of the target cell (Berkner, 1992, *Curr. Topics Microbiol. Immunol.*, 158: 39-66; Boviatsis et al., 1994, *Human Gene Therap.*, 5: 183-191) and thus provide temporal recombinant gene expression from an extra-chromosomal element for a period of several weeks to a month.

Replication-defective recombinant viruses and plasmid-derived amplicons derived from herpes virus vectors have been developed for gene delivery into cells and tissues (Leib et al., 1993, *Bioessays*, 15: 547-54; Boviatsis et al., 1994, *Human Gene Therap.*, 5: 183-191). Both herpes-derived gene delivery vectors are relatively nonpathogenic to neural tissues and can mediate transgene expression in a substantial number of neurons and other cell types. The recombinant herpes vectors have the distinct advantage that they can enter a latent state in some neuronal cells and thus could potentially mediate stable transgene expression. Adeno-associated virus (AAV) has several desirable characteristics as a vector for gene therapy (Kotin, R.M., 1990, *Proc. Natl. Acad. Sci. USA*, 87: 2211-5; Muzyczka, N., 1992, *Curr. Topics Microbiol. Immunol.*, 158: 97-129). AAV is nonpathogenic in both humans and animals and has a broad host range including human, primate, canine and murine. Its ability to infect and integrate into nondividing cells with high frequency makes it a desirable vector for transfecting post-mitotic epithelia of the inner ear (Kaplitt et al., 1994, *Nature Genet*, 8: 148-154). AAV integration is stable; AAV remained stably integrated in the genome of transformed cells through 150 passages. Thus, AAV and AAV-derived vectors are preferred for use in the present invention in the transformation of cells of the inner ear.

Where a viral vector is used to accomplish target cell transformation, the viral vector is preferably a replication-deficient virus. Where a replication-deficient virus is used as the viral vector, infective virus particles containing either DNA or RNA corresponding to the desired therapeutic gene product can be produced by introducing the viral construct into a recombinant cell line which provides the missing components essential for viral replication in trans. Preferably, transformation of the recombinant cell line with

the recombinant viral vector will not result in production of replication-competent viruses (e.g., by homologous recombination of the viral sequences of the recombinant cell line into the introduced viral vector).

Methods for production of replication-deficient viral particles containing a nucleotide sequence of interest are well known in the art and are described in, for example, Rosenfeld et al., *Science* 252:431-434, 1991 and Rosenfeld et al., *Cell* 68:143-155, 1992 (adenovirus); USPN 5,139,941 (adeno-associated virus); USPN 4,861,719 (retrovirus); and USPN 5,356,806 (vaccinia virus).

The vector for transformation is composed of (in the case of a nonviral vector) or derived from (in the case of recombinant viral vectors) a DNA construct. Preferably, the DNA construct contains a promoter to facilitate expression of the DNA of interest within the inner ear cell. Preferably the promoter is a strong, eukaryotic promoter.

Exemplary eukaryotic promoters include promoters from cytomegalovirus (CMV), mouse mammary tumor virus (MMTV), Rous sarcoma virus (RSV), adenovirus, herpes simplex virus (HSV) (e.g., HSV thymidine kinase promoter), and SV40. More specifically, exemplary promoters include the Ad 2 major late promoter (Wong et al. 1986 *J. Virol.* 60(1):149-56), the promoter from the immediate early gene of human CMV (Boshart et al., *Cell* 41:521-530, 1985) and the promoter from the long terminal repeat (LTR) of RSV (Gorman et al. 1982 *Proc. Natl. Acad. Sci. USA* 70:6777-6781). Of these promoters, the CMV and Ad 2 major late promoters are especially preferred.

Other components of constructs suitable for use include a marker(s) (e.g., an antibiotic resistance gene (such as an ampicillin resistance gene), β-galactosidase or green fluorescent protein (GFP)) to aid in selection of cells containing the construct, an origin of replication for stable replication of the construct in a bacterial cell (preferably, a high copy number origin of replication), a nuclear localization signal, or other elements which facilitate production of the DNA construct, the protein encoded thereby, or both. Where the therapeutic gene product is to be secreted (e.g., into the scala vestibuli, scala tympani, or scala

media), the DNA of interest is preferably operably linked to a secretion signal. Such secretion signals can provide for delivery of the gene product of interest into an inner ear lumen (i.e., the cochlear duct) and/or into a fluid of the inner ear (e.g., the endolymph, perilymph, and or cortilymph).

For eukaryotic expression, the construct should contain at a minimum a eukaryotic promoter operably linked to the DNA of interest, which is in turn operably linked to a polyadenylation sequence. The polyadenylation signal sequence may be selected from any of a variety of polyadenylation signal sequences known in the art. Preferably, the polyadenylation signal sequences are the polyadenylation signal sequences of the SV40 late and/or early genes. The construct may also include one or more introns, which can increase levels of expression of the DNA of interest. Any of a variety of introns known in the art may be used. For example, the human β-globin intron can be inserted in the construct at a position 5' to the DNA of interest to provide enhanced expression.

The DNA of interest can be inserted into a construct so that the therapeutic protein is expressed as a fusion protein. For example, the therapeutic protein can be a portion of a fusion protein having β-galactosidase or a portion thereof at the N-terminus and the therapeutic protein at the C-terminal portion. Or, for example, the therapeutic protein (or a portion thereof) can be fused to green fluorescent protein (or a portion thereof). Methods for production of such fusion proteins are well known in the art (see, for example, Sambrook et al. *Molecular Cloning: A Laboratory Manual*, 2nd Ed., 1989, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY; Kain et al. 1995 *Biotechniques* 19:650-655; and Clontech Laboratories, Inc., *Technical Service Protocol* #PT2040-1, Version #PR64559, each of which are hereby incorporated by reference with respect to methods and compositions for production and expression of fusion proteins). Production of a fusion protein can facilitate monitoring of therapy, e.g., through detection of the fusion protein from a sample of perilymph fluid where the

therapeutic protein is secreted into the perilymph of the cochlea.

It may also be desirable to produce altered forms of the therapeutic proteins that are, for example, protease resistant or have enhanced activity relative to the wild-type protein. Further, where the therapeutic protein is a hormone, it may be desirable to alter the protein's ability to form dimers or multimeric complexes.

The construct containing the DNA of interest can also be designed so as to provide for site-specific integration into the genome of the target inner ear cell. Methods and compositions for preparation of such site-specific constructs are described in, for example, USPN 5,292,662, incorporated herein by reference with respect to the construction and use of such site-specific insertion vectors. Techniques for production of nucleic acid constructs for expression of exogenous DNA or RNA sequences in a host are known in the art (see, for example, Kormal et al., *Proc. Natl. Acad. Sci. USA*, 84:2150-2154, 1987; and Sambrook et al., supra, each of which are hereby incorporated by reference with respect to methods and compositions for eukaryotic expression of a DNA of interest).

Therapeutic gene products and conditions amenable to treatment by gene therapy of the inner ear

The DNA of interest can be any DNA encoding any gene product, e.g., protein, RNA molecule, or other gene product, for which therapy is desirable. For example, protein therapy (i.e., through intracellular or secretory protein expression) is appropriate in treating a subject having an inherited or acquired disease associated with a specific protein deficiency. Such protein-deficient states are amenable to treatment by replacement therapy, i.e., expression of a protein to restore at least normal protein expression levels.

The DNA of interest can encode proteins involved in the development of inner ear structures. For example, the DNA of interest can encode gene product(s) to facilitate the development of functional hair cells. For example, where hearing loss is associated with dysfunctional hair cells or

loss of hair cells, it may be desirable to "genetically download" the differentiated hair cells to an undifferentiated state, then transform the de-differentiated hair cells with nucleotide sequences encoding gene product to facilitate the re-differentiation of the de-differentiated hair cells into functional hair cells, or to facilitate the development of functional hair cells from hair cell precursor or hair stem cells. Such application of the methodology of the invention can be useful where hearing loss is due at least in part to stereocilia retraction and scarring of the tectorial plate associated with a region in the cochlea devoid of functional hair cells.

Alternatively, the subject may have or be susceptible to a condition that is amenable to treatment by expression or over-expression of a protein which is either normally present in a healthy subject or is foreign to the subject. For example, antibiotic or antiviral protein therapy may be desired for treatment of a mammalian subject having a viral, bacterial, fungal, and/or parasitic infection of the inner ear, particularly where the infection is chronic, i.e., persisting over a relatively long period of time. Autoimmune disorders can be treated by expression of an anti-inflammatory agent, cytokine, anti-antibody or fragment thereof, or other gene product(s) that can be useful in treating such disorders. In addition, bone formation in the cochlear following meningitis (osteoneogenesis) can be treated or prevented by expression of gene product that inhibit such bone formation processes. Expression of nerve growth factor (NGF) may be desired for regeneration of the auditory nerve. Auditory nerve regeneration is desirable where, for example, hearing loss is associated with regression of neurites of the cochlear nerve from the spiral organ of Corti in a direction toward the cell body of the spiral ganglion (see Fig. 3). Expression of nerve growth factors can facilitate neurite growth toward the organ of Corti and the auditory nerve cells, or retard degeneration of auditory dendrites, to restore cochlear function, thereby improving hearing or stabilizing hearing loss.

Expression of the DNA of interest can result in production of a gene product that is either maintained within the transformed cell or secreted by the transformed cell to achieve the desired effect. For example, structural proteins will generally be maintained as intracellular components of the transformed cell, while gene products that achieve their desired effect through antibiotic activity are generally secreted into a space or fluid of the inner ear. Gene products for which secretion may be desirable include gene products that affect inner ear fluid osmolarity, fluid viscosity, promote contractility, affect fluid pH, act as anti-inflammatory agents, and/or affect ion channels (e.g., sodium, potassium and/or calcium ion channels).

The DNA of interest is preferably obtained from a source of the same species as the subject to be treated (e.g. human to human), but this is not an absolute requirement. DNA obtained from a species different from the subject can also be used, particularly where the amino acid sequences of the proteins are highly conserved and the xenogeneic protein is not highly immunogenic so as to elicit a significant, undesirable antibody response against the protein in the host.

Exemplary, preferred DNAs of interest include DNA encoding nerve growth factors, (e.g., NGF, BDNF, NT-3, NT4/5, p70) structural proteins (e.g., actin, myosin, dystrophin, dystrophin-related protein (DRP), MERLIN, and neurofibronin), electrolyte channels (e.g., for the potassium, calcium and sodium ions), mechanotransduction proteins water channels, and transcription factors (e.g., homeobox and paxeal transcription factors). Preferably, the subject is a human subject and the DNA expressed encodes a human protein (e.g., human nerve growth factor). Table 1 provides a list of exemplary proteins and protein classes which can be delivered by the inner ear gene therapy of the invention.

Table 1. Exemplary Gene Products, Proteins and Protein Classes for Delivery by Gene Therapy to the Inner Ear

Protein or Other Gene Product Class	Exemplary Proteins or Gene Products
Nerve growth factors	Nerve growth factor (NGF) Neurotrophin-3 Brain-derived neurotrophin factor p70 NT4/5
Structural proteins	Dystrophin Dystrophin-related protein Dystrophin-associated glycoprotein Myosins Actin MERLIN Neurofibronin
Ion Channels	Sodium channel Potassium channel Calcium channel
Cytokines/ Immunoregulatory Protein	Interleukins TGF-α, TGF-β GMCSF
Transcription Factors	Homeobox proteins PAX proteins POU3F4

Various disease conditions are amenable to treatment using the inner ear gene therapy of the invention. One skilled in the art can recognize the appropriate gene product (e.g., polypeptide or RNA) that should be produced for the treatment of specific disease conditions. A list of diseases and conditions associated with hearing loss (sensorineural or mixed) that are amenable to treatment according to the method of the invention are provided in Table 2. The genetics of hereditary hearing impairment is

reviewed in Mhatre and Lalwani, 1996, "Molecular Genetics of Deafness," *Update on Otology and Neurology, Part II*, Otolaryngol Clinic North Am. 29: 421-35, incorporated herein by reference with respect to description of the genetics of hearing impairment. Exemplary diseases amenable to treatment using the subject invention, and exemplary, appropriate proteins which can be used in treating these diseases, are shown in Table 3.

Table 2. Diseases Amenable to Treatment Using the Methodology of the Invention

AD=autosomal dominant AR=autosomal recessive
RP=retinitis pigmentosa XLD=X-linked dominant
XLR=X-linked recessive INHER.=Inheritance LINK.=Linkage

I. Hereditary Syndromic Hearing Impairment

DISORDER	PATHOLOGY	INHER.	LINK.	GENE	PROTEIN	REFERENCE
Albinism Deafness Syndrome (ADFN)	congenital deafness, patchy hypo and hyper pigmentation	XLR	Xq26.3			Shiloh et al. 1990 *Am. J. Hum. Genet.* 47:20-27
Alport's Syndrome	nephritis, ocular abnormalities	XLR AR AR	Xq22 2q35-q37 2q36-q37	COL4A5 COL4A4 COL4A3	collagen subuit	Barker et al., 1990, *Science*, 248:1224-1227 Mochizuki et al., 1994, *Nat. Genet.*, 8:77-81 Lemmink et al., 1994, 3:1269-1273
Branchio-Oto-Renal Syndrome (BOR)	branchial remnants, ear abnormalities	AD	8q12-q14			Kumar et al., 1992, *Hum. Mol. Genet*, 1:491-495
Cockayne Syndrome	cachetic dwarfism, premature aging	AR	2q21	ERCC	DNA helicase	Weeda et al., 1990, *Cell*, 62:777-791
Crouzon Syndrome	craniosynostosis	AD	10q25-q26	FRFR2	growth factor receptor	Reardon et al., 1991, *Genomics*, 11:885-894

DISORDER	PATHOLOGY	INHER.	LINK.	GENE	PROTEIN	REFERENCE
Charcot-Marie Tooth (CMT) disease CMT1A	peripheral neuropathy, cerebellar degeneration	AD	17p11.2	PMP-22	myelin protein	Valentijn et al., 1992, *Nat. Genet.*, 2:288-291
CMT1B		AD	1q22	MPZ		
CMT4A		AR	8q13-q21.1			Su et al., 1993, *Proc. Natl. Acad. Sci. USA*, 90:10856-10860
CMT-X		XLD	Xq11-q21			Ben Othmane et al., 1993, *Hum. Mol. Genet.*, 2:1625-1628
						Le Guern et al., 1994, *Neuromuscul Disord.*, 4:463-464
Hunter Syndrome	accumulation of mucopolysaccharides in brain and other tissues	XLR	Xq28	IDS	lysosomal enzyme	Palmieri et al., 1992, *Genomics*, 12:52-57
Hurler Syndrome	accumulation of mucopolysaccharides in brain and other tissues	AR	4p16.3	IDUA	lysosomal enzyme	Scott et al., 1992, *Hum. Mutat*, 1:103-108
Neurofibromatosis type 2 (NF2)	bilateral acoustic neuromas	AD	22q	NF2	MERLIN, a trans-membrane protein	Rouleau et al., 1993, *Nature*, 363:515-521
Norrie's Disease	congenital blindness, cognitive defects	XLR	Xp11.3	ND gene	ND protein (NDP)	Berger et al., 1992, *Nat. Genet.*, 2:84
OASD	ocular albinism and late onset sensorineural deafness	XLR	Xp22.3			Winship et al., 1993, *Genomics*, 18:444-445

DISORDER	PATHOLOGY	INHER.	LINK.	GENE	PROTEIN	REFERENCE
Osteogenesis Imperfecta	primary defect of connective tissue and bone	AD	7q21.3 17q21.3	COL1A2 COL1A1	collagen subunit collagen subunit	Myers et al., 1985, *Ann NY Acad. Sci.*, 460:482-485 Pope et al., 1985, *J. Med. Genet.*, 22:466-478
Piebaldism	hair and skin hypopigmentation	AD	4q12	KIT	mast/stem cell growth factor receptor	Giebel et al., 1991, *Proc. Natl. Acad. Sci. USA*, 88:8696-8699
Stickler Syndrome	mild spondyloepiphyseal dysplasia, osteoarthritis, and myopia	AD	12q12-q13.1 6p21.3	COL2A1 COL11A2	collagen subunit collagen subunit	Weaver et al., 1989, *Cytogenet Cell Genet.*, 51:1103 Vikkula et al., 1995, *Cell*, 80:431-437
Treacher-Collins Syndrome	craniofacial anomalies	AD	5q31.3			Dixon et al., 1992, *Hum. Mol. Genet.*, 1:249-253

DISORDER	PATHOLOGY	INHER.	LINK.	GENE	PROTEIN	REFERENCE
Usher's syndrome type I (severe)	congenital deafness, vestibular dysfunction and RP before puberty		14q			Kaplan et al., 1991, *Cytogenet Cell Genet.*, 58:1988(A 27446)
Ia						
Ib						
Ic						
type II	moderate to severe congenital deafness with RP in late teens		11q13	myosin		
IIa						
IIb						
type III			11p13	VIIA		Weil et al., 1995, *Nature*, 374:60-61
			1q41 not linked			Smith et al., 1992, *Genomics*, 14:995-1002
			3q			Kimberling et al., 1990, *Genomics*, 7:245-249
						Sankila et al., 1994, *Am. J. Hum. Gent.*, 55:A15

DISORDER	PATHOLOGY	INHER.	LINK.	GENE	PROTEIN	REFERENCE
Wardenburg's type I	pigmentary abnormalities with dystopia canthorum canthorum	AD	2q37	PAX3	transcription factor	Baldwin et al., 1992, *Nature*, 355:637-638; Tassabehji et al., 1992, Nature, 355:635-636
type II	without dystopia canthorum		3p14.1-p12.3	MITF	transcription factor	
type III	with dystopia canthorum and limb abnormalities		2q37	PAX3	transcription factor	Tassabehji et al., 1994, *Nat. Genet.*, 8:251-255; Hoth et al., 1993, *Am. J. Hum. Genet.*, 52:455-462

II. Hereditary Nonsyndromic Hearing Impairment

DISORDER	PATHOLOGY	INHER.	LINK.	GENE	PROTEIN	REFERENCE
DFN1	progressive deafness	XLR	Xq21-q22			Tranebjaerg et al., 1992, *Am. J. Hum. Genet.*, 51(suppl):A 47
DFN2	congenital deafness	XLR	X			Reardon et al., 1994, *Nat. Genet.*, 8:98-103
DFN3	Stapes fixation with perilyphatic gusher, progressive mixed deafness	XLR	Xq21.1	POU3F4	transcription factor	de Kok et al., 1995, *Science*, 267:685-688
DFN4	congenital deafness	XLD	Xp21.2			Lalwani et al., 1994, *Am. J. Hum. Genet*, 55:685-694
DFNA1 (Monge's deafness)	early onset (age 10) low frequency hearing loss, Costa Rican family	AD	5q31			Leon et al., 1992, *Proc. Natl. Acad. Sci. USA*, 89:5181-5184

DISORDER	PATHOLOGY	INHER.	LINK.	GENE	PROTEIN	REFERENCE
DFNA2	early onset, high frequency hearing loss, Indonesian family	AD	1p31-32			Coucke et al., 1994, *N. Engl. J. Med.*, 331:425-431
DFNA3	early onset, high frequency hearing loss, French family	AD	13q12			Chaib et al., 1994, *Hum. Mol. Genet*, 3:2219-2222
DFNA4	progressive hearing loss that begins in the second decade, American family	AD	19q13			Chen et al., 1995, *Hum. Mol. Genet.*, 4:1073-1076
DFNA5	early onset hearing loss, Dutch family	AD	7p15			Valentin et al., 1992, *Nat. Genet.*, 2:288-291
DFNA6	progressive low frequency hearing loss, American (southeast) family	AD	4p15.3			Lesperance et al., 1995, *Hum. Mol. Genet.*, 4:1967-1972
DFNA7	progressive high frequency hearing loss, Norweigen family	AD	1q21-q23			Tranebjaerg et al., 1995, *The Molecular Biology of Hearing and Deafness*, Bethesda, MD, October 6-8
DFNA8	moderate to severe hearing loss, Austrian family	AD	15q15-q21			Kirshhofer et al., 1995, *The Molecular Biology of Hearing and Deafness*, Bethesda, MD, October 6-8, 1995
DFNA9		AD	14q12-q13			Manolis et al., 1996 *Hum. Mol. Genet*
DFNA10		AD	6q22-q23			O'Neill et al., 1996 *Hum. Mol. Genet*
DFNB1 (NSRD1)	congenital deafness, Northern Tunisian family	AR	13q12			Guilford et al., 1994, *Nat. Genet.*, 6:24-28
DFNB2	congenital deafness, Tunisian family	AR	11q13.5			Guilford et al., 1994, *Hum. Mol. Genet.*, 3:989-993

DISORDER	PATHOLOGY	INHER.	LINK.	GENE	PROTEIN	REFERENCE
DFNB3	congenital deafness, Indonesian family	AR	17p-17q12			Friedman et al., 1995, *Nat. Genet.*, 9:86-91
DFNB4	congenital deafness	AR	7q31			Baldwin et al., 1995, *Hum. Mol. Genet.*, 4:1637-1642
DFNB5	consanguineous nuclear families from India with congenital hearing loss	AR	14q12			Fukushima et al., 1995, *Genome Research*, in press
DFNB6	(as above)	AR	3p21-p14			Fukushima et al., 1995, *Hum. Mol. Genet.*, 4:1643-1648
DFNB7	(as above)	AR	9q13-q21			Jain et al., 1995, *Hum. Mol., Genet.*, 4:2391-2394
DFNB8		AR	21q22			Veske et al., 1996, *Hum. Mol. Genet.*, 5:165-168
pending		AR	2p22-p23			Chaib et al., 1996, *Hum. Mol. Genet.*, 5:155-158

III. Mitochondrial Inheritance

DISORDER	PATHOLOGY	INHER.	LINK.	GENE	REFERENCE
Aminoglycoside induced deafness	susceptibility to aminoglycoside	mito		12S rRNA A->G (base 1555)	Prezant et al., 1993, *Nat. Genet.*, 4:289-294
Diabetes mellitus and hearing loss	diabetes and progressive deafness	mito		10.4 kb deletion $tRNA_{leu}$	Ballinger et al., 1992, *Nat. Genet.*, 1:11-15 van den Ouweland et al., 1994, *Diabetes*, 43:746-751

tRNA-Ser(UCN)	hearing loss of varying severity			T7445C	Reid et al., 1994 *Hum. Mutat.* 3: 243-7
tRNA-Ser(UCN)	hearing loss, ataxia and myoclonus			7472insC	Tiranti et al., 1995 *Hum. Mol. Genet.* 4:2159-63

IV. Other Conditions or Diseases Amenable to Treatment Using the Methodology of the Invention

General Category of Condition/Disease	Specific Disease/Conditions
Disorders of the acoustic (vestibular) nerve (cranial nerve VIII)	Brain stem lesion Acoustic neuroma Schwannomas Vestibular neuronitis
Inherited neuropathies	Refsum's disease (HMSN type IV)
Sensorineural loss accompanying aging	Presbycusis
Infections	viral infections (e.g., CMV, mumps) bacterial infections (e.g., meningococcal meningitis, meningitis caused by *Hemophilus influenzae* or *Streptococcus pneumoniae*, syphilis) Lassa fever
Osmotic disorders	Hydrops
Vestibular disorders	Ménière's disease Vertigo
Autoimmune disorders	Cogan's Syndrome autoimmune hearing loss system lupus erythmatosa (SLE) rheumatoid arthritis
Environmental exposure	noise radiation therapy trauma

General Category of Condition/Disease	Specific Disease/Conditions
Ototoxic pharmaceuticals	aminoglycoside antibiotics streptomycin gentamicin netilmicin neomycin kanamycin amikacin tobramycin ceftriaxone cisplatin (chemotherapy) deferoxamine (treatment of iron overload) ethacrynic acid (loop diuretic)
Cerebrovascular diseases	arterial occlusion of the internal auditory artery
Etiology unknown	Ménière's disease

Table 3. Exemplary Diseases Amenable to Treatment by Gene Therapy of the Inner Ear and Exemplary Therapeutic Gene Products for Treatment

Disease or Condition	Exemplary Therapeutic Gene Products for Treatment of the Disease or Condition
Auditory nerve degeneration	Nerve growth factor (NGF) Neurotrophin-3 (NT-3) Brain-derived neurotrophic factor (BDNF) NT4/5 p70
Defects in hair cell differentiation	
Damaged hair cells	Factors for hair cell regeneration??? Retinoic acid
Cochlear Implantation	Factors that enhance neurite growth (e.g., NGF, neurotrophin-3, BDNF, NT4/5, p70)

Of particular interest is the use of the invention to treat or prevent hearing loss associated with exposure to ototoxic pharmaceuticals (e g., through the expression of nerve growth factors (e.g., NGF, NT-3, BDNF, NT 4/5, and p70)) and to treat or prevent other conditions associated with auditory nerve degeneration or damage.

Numerous proteins desirable for therapy for the inner ear are well known in the art, and the DNA encoding these proteins has been isolated and are available from Genbank

and/or have been described in the scientific literature. Table 4 provides a list of exemplary gene products and their corresponding Genbank accession numbers.

Table 4. Exemplary Gene Products for Expression in The Inner Ear

I. Transcription Factors

Gene Product	Genbank Accession No.
Human mRNA for transcription factor, Lbx1	X90828
Human HOXB6 gene, Hoxb-6 LPM enhancer-like sequence	U19111
Human G17 gene	X80700
Human engrailed protein (EN2) gene, 5' end	HUMEN2AO
Human HOX A1=class I homeobox {fragment M3, homeodomain}	S69025
Human HOX B6=class I homeobox {fragment M2, homeodomain}	S69023
Human HPX-153 homeobox (mRNA)	X76978
Human HPX-6 (mRNA)	X74863
Human Brain 4 (mRNA)	X82324
Human PAX3B=transcription factor	S69370
Human PAX3A=transcription factor	S69369

II. Ion Channels

Gene Product	Genbank Accession No.
Human voltage-gated chloride ion channel CLCN5	X91906
Human delayed rectifier potassium channel (Isk) gene	L33815
Human potassium channel protein (HPCN3) gene	M55515
Human potassium channel (HPCN2) (mRNA)	M55514
Human potassium channel (HPCN1) (mRNA)	M55513

Gene Product	Genbank Accession No.
Human gamma subunit of epithelial amiloride-sensitive sodium channel (mRNA)	X87160
Human beta subunit of epithelial amiloride-sensitive sodium channel	X87159

III. Structural Proteins

Gene Product	Genbank Accession No.
zd72b06.r1 Soares fetal heart NbHH19W Human cDNA clone 346163 5' similar to gb:X13839 ACTIN, AORTIC SMOOTH MUSCLE (HUMAN)	W78033
ma39b12.r1 Soares mouse p3NMF19.5 Mus musculus cDNA clone 313055 5' similar to gb:J00068 ACTIN, ALPHA SKELETAL MUSCLE (HUMAN)	W10590
Human nonmuscle myosin heavy chain-A (MYH9) (mRNA)	M69180
Human beta-actin (mRNA)	X00351
Mouse dystrophin (mRNA)	M68859
Human dystrophin-related protein 2 (DRP2) (mRNA)	U43519
yv71a07.r1 Human cDNA clone 248148 5' similar to gb:M18533 DYSTROPHIN (HUMAN)	N78018
Human (YAC 3'-19) DMD gene for dystrophin	X75580
Human (YAC 5'-5) DMD gene for dystrophin	X75801
Human cDNA clone HE6WCR175 3'	H54881
Human cDNA clone 44648 5' similar to gb:X05608_cds1 NEUROFILAMENT TRIPLET L PROTEIN (HUMAN)	h06940
Human myosin VIIA (USH1B) (mRNA)	U39226
Mus musculus unconventional myosin VI (sv) (mRNA)	U49739

Where the DNA encoding a protein of interest has not been isolated, this can be accomplished by various, standard protocols well known to those of skill in the art (see, for

example, Sambrook et al., *ibid*; Suggs et al., *Proc. Natl. Acad. Sci. USA* 78:6613-6617, 1981; USPN 4,394,443; each of which are incorporated herein by reference with respect to identification and isolation of DNA encoding a protein of interest). For example, genomic or cDNA clones encoding a specific protein can be isolated from genomic or cDNA libraries using hybridization probes designed on the basis of the nucleotide or amino acid sequences for the desired gene. The probes can be constructed by chemical synthesis or by polymerase chain reaction (PCR) using primers based upon sequence data to amplify DNA fragments from pools or libraries (USPNs 4,683,195 and 4,683,202). Nucleotide substitutions, deletions, additions, and the like can also be incorporated into the polynucleotides, so long as the ability of the polynucleotide to hybridize is not substantially disrupted. (Sambrook et al. *ibid*). The clones may be expressed or the DNA of interest can be excised or synthesized for use in other constructs. If desired, the DNA of interest can be sequenced using methods well known in the art.

Transformation and Administration

In general, transformation is accomplished by introducing the nucleotide sequence of interest (e.g., the DNA of interest contained in a construct that is formulated in a chemical vector solution or as a viral vector) directly into the inner ear, and may be inserted into or near the desired inner ear structure or cell (e.g., directly into the cochlear or vestibular structure). Introduction of the DNA of interest can be accomplished by any means, generally by injection or infusion. In a preferred embodiment, the DNA of interest is delivered to the inner ear *in vivo* by infusion, preferably using an osmotic minipump. Implantation of an osmotic minipump and its use in the delivery of pharmacologic agents to the brain, kidney, and guinea pig cochlea have been described (Schindler et al., 1977 *Arch. Otolaryngol*, 103:691-699; Nau, 1985 *Toxical. Appl. Pharmacol.*, 80:243-250; Sendelbeck et al., 1985 *Brain Res.*, 328:251-258; Ruers et al., 1986 *Transplantation*, 41:156-161; Davies et al., 1994 *Am. J. Otology*, 15:757-761, Schindler et al., 1995 *Am. J. Otology*,

16:304-309; and Shah et al., 1995 *Am. J. Otology*, 16:310-314, each of which are incorporated herein by reference with respect to the description of the implantation and use of such osmotic minipumps). An exemplary osmotic minipump is shown in Fig. 7.

Introduction of the DNA of interest into the inner ear can be accomplished delivery through the round or oval window of the inner ear. Where the cells to be transformed are cells of the cochlea or cochlear structures, it may be preferable to introduce the DNA of interest into the inner ear through the round window. Where the cells to be transformed are vestibular cells, or where the subject is undergoing stapes-related surgery, it may be preferable to introduce the DNA into the inner ear via the oval window.

Alternatively, cochlear cells can be transformed *in vitro*, and the transformed cochlear cells subsequently implanted into the subject's cochlea. Isolation, maintenance, and implantation of cochlear cells are well known in the art.

The form of the preparation for transformation of the inner ear cells will depend upon several factors such as the cochlear cell targeted for gene transfer and whether a biological or nonbiological vector is employed. The vector solution can also contain therapeutic compounds (e.g, nerve growth factors, anti-inflammatory agents, antibiotic agents) in addition to the DNA of interest, as well as compounds to adjust, for example, the pH, osmolarity, and/or viscosity of the vector solution. The preparation can additionally contain compounds that facilitate entry of the nucleic acid of interest into the inner ear cells such as lipofectin, permeability-enhancing agents (e.g., detergents), or other transformation-enhancing agents. Where the vector is a viral vector, the preparation can also include a co-infecting virus to facilitate infection and transformation. Where the DNA of interest is administered in a recombinant viral vector, e.g., an AAV vector, the vector solution is preferably normal saline.

The amount of DNA and/or number of viral particles administered will vary greatly according to a number of factors including the susceptibility of the inner ear cells to

transformation, subject-dependent variables such as age, weight, sensitivity or responsiveness to therapy, susceptibility of targeted inner ear cells to transformation, the levels of protein expression desired, and the condition to be treated. For example, where a recombinant AAV vector is used, the total delivered viral dosage can be in the range of 1 virus per 5 cochlear cells, preferably 1 virus per 10 cochlear cells, more preferably 1 virus per 20 cochlear cells or less. Generally, the amounts of DNA for transformation of human inner ear cells can be extrapolated from the amounts of DNA effective for gene therapy in an animal model. For example, successful transformation of the inner ear cells of a guinea pig was accomplished using 10^5 particles of AAV containing the DNA of interest at an moi (multiplicity of infection) of approximately 1:20 (1 viral particle per 20 cochlear cells).

The amount of DNA and/or viral particles necessary to accomplish transformation of inner ear cells will decrease with an increase in the efficiency of the transformation method used. In general, the amount of DNA and/or the number of infectious viral particles is an amount effective to infect the targeted inner ear cells or structure, transform a sufficient number of inner ear cells, and provide for expression of desired or therapeutic levels of the protein or other gene product. Where transformation is transient (e.g., the DNA of interest is maintained for some period as an extrachromosomal element), the time period over which expression is desired may also be taken into consideration. The desired number of copies (e.g., copy number) of the DNA of interest in the cell may additionally be taken into account in determining the amount of DNA and/or number of viral particles to be delivered to the subject, and such may be adjusted as desired to, for example, achieve varying levels of gene product expression.

Transformation can be accomplished such that expression of the gene product of interest is either transient, inducible, or stable. For example, where the DNA of interest is present in the transformed cell as an extrachromosomal element (e.g., as with AAV vectors),

expression of the gene product is generally transient. Inducible expression can be achieved so that expression of the gene product of interest is induced only in the presence of some signal that is, for example, specific to a certain type of cell of the inner ear (e.g., is only expressed in cochlear cells or a specific type of cochlear cell due to the presence of a cell-specific or tissue-specific transcription factor in the transformed cell). Alternatively, gene product can be inducible by the presence of an extracellular factor that can be introduced at the same time as the transforming vector solution is introduced into the inner ear, and/or subsequent to inner ear cell transformation. Stable expression of the gene product can be achieved by, for example, introduction of the DNA of interest in a vector to provide for stable genomic integration into the inner ear cell and expression of the gene product from the DNA of interest via a constitutive promoter.

Where expression of the gene product of interest is transient, expression can be maintained in the inner ear cell for a period ranging from several days to several months or years, e.g., for 6 months to 1 year, for 4 months to 6 months, for 2 weeks to 8 weeks, or for as little as one week or a few days (e.g., 3 to 5 days, or 1 to 3 days). Transient expression of the gene product of interest may be desirable where the subject is being exposed to the therapeutic regimen for the first time (e.g., where it is desirable to monitor the responsiveness and/or sensitivity of the subject), or where expression is desired only over a specific period (e.g., for a period after cochlear implantation without permanent expression, or for a period during a specific stage of development). The period of transient expression can be adjusted by , for example, adjusting the transformation protocol to achieve a desired number of transformed cells or, where a viral vector is used, by adjusting aspects of the vector associated with maintenance in a cell (e.g., replication functions or other functions associated with vector stability and/or copy number).

The actual amounts of DNA and/or number of infectious viral particles required can be readily determined based upon

such factors as the levels of protein expression achieved in cell lines *in vitro*, and the susceptibility of the inner ear cells to transformation.

Identification of Subjects for Inner Ear Gene Therapy

Any patient having a sensorineural or mixed hearing loss and/or vestibular dysfunction can be treated according to the method of the invention. The type of hearing loss suffered by the candidate individuals can be determined using various methods well known in the art such as pure tone air- and bone-conduction audiometry, impedance (or immittance) batteries, and speech audiometry. In measuring hearing, stimuli are presented to the ear through air conduction (i.e., by earphones) or through bone conduction, in which a vibrator is placed on portions of the skull. A pure tone air-conduction test measures hearing impairment in the outer, middle, and inner ear, whereas bone conduction conducts sound directly to the inner ear. Those individuals having a hearing loss associated with the inner ear (e.g., a sensorineural or mixed hearing loss) are amenable to the method of treatment according to the invention.

A pure tone audiometer can be used to determining the degree and type of hearing loss and produces an audiogram that measures hearing sensitivity as a function of frequency. Simple and reliable screening can be accomplished using a hand-held Welch Allyn audioscope, an otoscope with a special attachment that delivers a 40 dB tone at frequencies form 500 to 4,000 Hz. When compared with pure tone audiometry, this simple test has a 91 to 97 percent sensitivity and 69 to 85 percent specificity. The range from -10 dB to 25 dB on the audiogram is considered within normal limits. A greater than 25 dB loss constitutes a hearing impairment. A 25 dB loss is a mild impairment, a 40 dB loss is a moderate impairment, and a 70 dB loss is a severe impairment.

Impedance (or immittance) measurements evaluate the integrity and performance of the peripheral auditory system. The basic battery of immittance measurements include static compliance, measurement of the acoustic reflex, and tympanometry, the most sensitive indicator of middle-ear

function available. Impedance evaluation is useful in distinguishing ossicular-chain discontinuity from otosclerosis.

Speech audiometry measure overall hearing performance for functional speech stimuli. Two aspects of auditory performance are assessed: speech reaction threshold (SRT) and speech discrimination. The SRT is the level at which the patient recognizes two-syllable spondaic words, which have equal stress on both syllables, 50 percent of the time. Speech discrimination measures the subject's ability to understand speech presented at 30 to 40 dB above the patient's SRT. For example, subjects with presbycusis lose the ability to differentiate consonants, in part because these sounds are usually of a higher frequency. Thus, speech discrimination deteriorates. The patient is scored according to the percentage of speech understood (e.g., 50 percent speech discrimination indicates that the patient understands 50 percent of speech heard). If difficulty in speech discrimination is suspected, audiological testing is recommended. Questioning the patient about he ability to understand speech may aid in identifying discrimination difficulty.

In addition, subjects having tinnitus, commonly described as a ringing in the ears, may also be amenable to treatment using the method of the invention. Tinnitus is a form of hearing impairment that occurs in 10 to 37 percent of the elderly population and can result from any disease or injury that affects the auditory system. All ototoxic effects are signaled by tinnitus, but this condition may also result from impairment of any part of the auditory pathway and have a vascular, muscular, or hormonal origin.

In general, the method of the invention can be used to treat sensorineural and mixed hearing losses associated with exposure to ototoxic agents, genetic defects associated with defects in the structures and components of the inner ear (e.g., cochlear structures (e.g., auditory hair cells, spiral ganglion, scala vestibuli, Hansen's cell, spiral organ of Corti, stria vascularis, and scala tympani), endolymph, perilymph, semicircular ducts, ampullae, utricle, and

saccule), vestibular disorders, inheritable hearing loss, infectious disease (e.g, mumps), cancerous growths, exposure to noise levels of greater than 85 to 90 dBA (decibels to the a scale, which approximates response of the human ear) for more than 8 hr intervals, exposure to hazardous materials associated with decrements of audiovisual performance, and/or hearing losses and vestibular disorders associated with age.

In addition, individuals who are at risk of developing a hearing loss may be treated according to the method of the invention prior to the actual onset of hearing loss. For example, the method of the invention can be used as a preventive or prophylactic measure to avoid, prevent, or reduce sensorineural and/or mixed hearing loss in individuals who are or will be receiving therapy with ototoxic agents, have a genetic defect associated with hearing loss or the development of hearing loss, have a family history of heritable hearing loss, have or are susceptible to a infectious disease (e.g., mumps) or tumorous growth associated with hearing loss, are exposed to or have been exposed to noise level of greater than 85 to 90 dBA (decibels to the a scale, which approximates response of the human ear) for more than 8 hr intervals, are exposed to hazardous materials associated with decrements of audiovisual performance; and/or exhibit early signs of hearing loss associated with age.

The methodology of the invention can also be used in conjunction with cochlear implantation. Methods for cochlear implantation are known in the art (see, for example, Schindler et al. 1977 *Arch. Otolaryngol.* 103:691-699; Schindler et al. 1993 *A.J.O.* 14:263-272; Souliere et al. 1994 *Otolaryngol. Clin. North. Am.* 27:533-536; Parkin et al. 1994 *E.N.T. J.* 73:156-158). For example, an osmotic minipump can be put in place at the time of cochlear implantation to provide for infusion of nerve growth factor and/or infusion of a transforming vector that encodes a nerve growth factor (e.g., NGF, NT-3, BDNF, NT4/5, p70, etc.) to facilitate the success of the cochlear implant through induction of nerve growth toward the electrode. The methodology of the invention can be used in conjunction with conventional methods of treatment of hearing loss.

In addition, the methods and compositions of the invention can be used to provide transgenic animal models useful as models of human hearing, development of inner ear structures (e.g., cochlear and/or vestibular development), and/or hearing conditions or disorders. For example, the methods of the invention can be used to transform inner ear cells of a nonhuman subject, e.g., a mouse, primate, chick, chick embryo, guinea pig, frog, bullfrog, or other animal useful as a model of hearing. The methods of the invention can be applied to transform the inner ear cells of animals presently accepted as models for hearing. Such conventional animal models for hearing are described in, for example, Davies et al. 1994 *Am. J. Otology* 15:757-761; Leake-Jones et al. 1982 *Hear Res.* 8:225-246; Leake et al. 1988 *Hear Res.* 33:11-34; Avraham et al. 1995 *Nature Genet* 11:369-375; and Gibson et al. 1995 *Nature* 374:62-64, each of which is incorporated by reference with respect to description of art-accepted animal models of hearing and hearing disorders.

Assessment of Therapy

The ability of the transformed inner ear cells to express the DNA of interest can be assessed by various methods known in the art. For example, therapy can be assessed using the conventional basic audiological evaluation composed of pure tone air- and bone-conduction audiometry, the impedance battery, and speech audiometry as described above to identify subjects amenable to treatment using the method of the invention. Prevention of hearing loss, or stabilization or an improvement in auditory acuity after treatment (relative to auditory acuity before treatment) is indicative of a subject's positive responsiveness to therapy. Auditory acuity can be measured by use of an instrument called an audiometer, which consists of an earphone connected to an electronic oscillator capable of generating pure tones ranging from low to high frequencies. Positive responsiveness to therapy can also be associated with, for example, prevention of osteoneogenesis (e.g., following meningitis), amelioration of vestibulopathy, tinnitus, and/or enhanced rehabilitation with cochlear implantation.

Where the therapeutic gene product or other gene product of interest is secreted into the endolymph or perilymph of the inner ear, successful transformation of inner ear cells and expression of the DNA of interest can also be assessed by, for example, assaying for the presence of the gene product in the endocochlear fluid. For example, a sample of fluid can be obtained from the inner ear, and expression of a protein of interest detected by performing an ELISA on the sample using an antibody which specifically binds the protein encoded by the DNA of interest. The ELISA can be performed either qualitatively or quantitatively. The ELISA assay, as well as other immunological assays for detecting a protein in a sample, are described in Antibodies: a Laboratory Manual (1988, Harlow and Lane, ed.s Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).

Alternatively, or in addition, the efficacy of the protein therapy can be assessed by testing a sample of fluid from the inner ear for an activity associated with the therapeutic protein (e.g., an enzymatic activity). For example, where the therapeutic protein has antimicrobial activity, the efficacy of therapy can be tested by examining the ability of the test sample to inhibit bacterial growth. Alternately, the efficacy of inner ear gene therapy can be assessed by monitoring the condition of the subject for improvement.

EXAMPLES

The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to carry out the invention and is not intended to limit the scope of what the inventors regard as their invention. Efforts have been made to ensure accuracy with respect to numbers used (e.g., amounts, temperatures, etc.), but some experimental error and deviation should be accounted for. Unless indicated otherwise, parts are parts by weight, molecular weight is weight average molecular weight, temperature is in degrees Centigrade, and pressure is at or near atmospheric.

Example 1: Construction of Recombinant AAV Vectors

Constructs for production of recombinant AAV vectors were prepared according to methods well known in the art (see, e.g., Sambrook et al, 1989, *Molecular Cloning: A Laboratory Manual*, 2nd Ed.). Briefly, the commercially available plasmid pUC19 (Yanish-Perron et al., 1985, *Gene* 33:103-19) was used as a "backbone" for construction. An x8mer NotI linker (New England Biolabs cat #1029) was cloned into the SmaI site of pUC19. This destroys the SmaI site, but generates in the process two SacII sites, one immediately on each side of the NotI linker. This vector was designated pN (NotI). A poly adenylylation signal, a 196bp fragment from the SV40 genome (nucleotides 2533-2729),was purified from an SV40 containing vector and, following the addition of BamHI linkers, was cloned into the unique BamHI site within pN. The fragment was oriented such that RNA polymerase transcribing from a promoter upstream of the NotI site passes through the NotI site and into the SV40 fragment, encountering the SV40 late gene polyadenylyation signal (the early gene polyadenylation signals appear in the opposite orientation. This vector was designated pNA (Adenylation signal).

Two promoters containing DNA fragments were cloned into pNA. A 180 bp region of the SV40 genome containing late viral protein gene 16S/19S splice donor and acceptor signals (obtained as a XhoI - PstI fragment from pLI (Okayama and Berg, 1983, *Mol. Cell Biol.* 3:280-9) was cloned into pNA to provide the appropriate RNA splicing signals as needed. This vector was designated pNAss (splice site).

The promoter-containing DNA fragments were cloned into pNA or pNAss. These fragments and vectors were all treated with T4 DNA polymerase prior to blunt end ligation. The herpes simplex thymidine kinase gene promoter was obtained from pXII (Boulter and Wagner, 1987, *Nucleic Acids Res.* 15: 7194) as a 925bp SaI - XhoI fragment. The human cytomegalovirus immediate early gene promoter and enhancer was obtained as a 619bp Tha I fragment from pCM5029 (Boshart et al., 1985, *Cell* 41:521-30). This was cloned into the HincII site of pUCI8 and subsequently recovered as a BamHI/HindIII fragment. The SV40 origin of DNA replication, enhancer, and

early promoter along with the SV40 late gene 16s/19s splice donor and acceptor sites was obtained as a 525bp HindIII - PstI from pLI (Okayama and Berg, 1983, supra). The adenovirus 2 major late promoter with a fused tri-partite leader containing a 5' splice donor signal with a 3' splice acceptor signal derived from an IgG gene was obtained as a 695bp XhoI - EcoRI fragment from p91023(B) (Wong et al. 1986 *J. Virol.* 60(1):149-56). These vectors were designated pNAss TK (Thymidine kinase promoter) , pNAssCMV (CMV promoter), pNASV (SV40 promoter) and pNAAd (adenovirus promoter). The *E-coli* β-gal gene from pC4AUGβ-gal (MacGregor et al., 1987, *Somat. Cell Mol. Genet.* 13:253-65) was excised as a 3530 bp EcoRI - XbaI fragment and following the addition of NotI linkers, cloned into the unique NotI site of each of the four expression vectors. These constructions were designated pSVβ, pCMVβ, pAdβ and pTKβ. Restriction endonuclease sites in parenthesis refer to sites lost during cloning.

An AAV vector for expression of the marker β-galactosidase (pTR-MAPβ) was constructed using procedures similar to those described above except the Ad 2 major late promoter was fused to the late gene 16s/19s splice donor/splice acceptor signal of SV40 (SD/SA SV40). The final plasmid construct contained *E. coli* β-galactosidase (β-gal) gene, driven by a late promoter of Adenovirus 2, flanked at both sides with wild type AAV terminal repeats (Fig. 6). The construct additionally contains polyadenylation signals of the SV40 late (pAR) and early (pAe) genes.

Additional constructs were prepared using similar techniques. A recombinant AAV vector for expression of neurotrophin-3 (NT-3) (Fig. 7) and a recombinant AAV vector for expression of brain-derived neurotrophin factor (BDNF) (Fig. 8) were also constructed using similar techniques and substituting the nucleotide sequence encoding NT-3 and BDNF for the nucleotide sequence encoding β-gal. A recombinant AAV vector encoding nerve growth factor (NGF) can be similarly constructed (Fig. 9).

In addition, constructs expressing β-gal (Fig. 10) or green fluorescent protein (GFP) (Fig. 11) from the CMV

immediate/early gene promoter were constructed according to methods well known in the art.

Example 2: Production of Recombinant AAV

Recombinant AAV for use in transformation of the cochlea was produced by co-transfecting 293 cells (human embryonic kidney cells, transformed with E1A-E1B region of Ad) with pTR-MAPβ (see Example 1) and a helper plasmid pIM29 (McCarty 92) carrying wild type AAV genome without terminal repeats. The same cells were also infected with Ad5 at a multiplicity of infection (moi) of 10. Recombinant AAV was harvested after 60 hr by freeze/thawing cells three times, centrifuging the lysed cells to remove cell debris, and heat inactivating adenovirus helper by incubating the lysate for 1 hr at 56°C. The AAV titer was determined in an infectious center assay according to methods known in the art. Briefly, cells in a well of 96-well dish, plated at 75% of confluence, were in-infected with varying dilutions of recombinant AAV, wild type AAV (moi 2), and Ad (moi 20). Thirty-six hours after infection, the cells were resuspended in media, harvested in phosphate-buffered saline (PBS), transferred onto a nylon filter 47 mm in diameter, and placed in to a microanalysis funnel with Frit support. The cells were then lysed *in situ* following a colony lift protocol (Sambrook et al., 1989, *Molecular Cloning, a Laboratory Manual, 2nd edition*). The filters were then hybridized to a P-labeled lacZ probe, the filters exposed, and cells infected with recombinant AAV were scored as small black dots.

Example 3: Transformation of the Inner Ear in a Guinea Pig Model

A total of fourteen adult male Hartley guinea pigs weighing between 400 g and 500 g were used to examine the ability of AAV to transform cochlear cells; eight guinea pigs were infused with recombinant AAV (test animals), two were infused with normal saline (mock infected controls), and four served as unoperated controls. Hartley guinea pigs wee used due to the relatively large size of their cochleae compared to the cochleae of mice and rats, and the ease of the surgical

manipulation in this species. Guinea pig care was in accordance with the guidelines of the Committee on Animal Care, University of California, San Francisco. Prior to surgery, Alzet osmotic minipumps (model 1007, Alza Corporation, Palo Alto, California) were prepared by connecting PE50 polyethylene tubing (Intramedic, Becton Dickinson and Company, Parsippany, New Jersey) to the flow moderator on the pump as previously described (Davies et al., 1994 *Am. J. Otology*, 15:757-761). The pump was filled with 100 µl PBS containing recombinant adeno-associated virus pTR-MLβ (10^6 viral particles/ml) which contained the bacterial β-gal sequence.

Surgery to implant the osmotic minipumps was performed by first anesthetizing the guinea pigs by intramuscular injection of ketamine (50 mg/kg) and xylazine (9 mg/kg). The tympanic bulla was exposed via a post-auricular incision and opened using a 1 mm diamond paste burr to allow visualization of the basal turn of the cochlea. A cochleostomy about 1 mm inferior to the round window was fashioned using a 0.5 mm diamond paste burr. The basilar membrane, visible through the cochleostomy, allowed confirmation of the correct positioning of the cochleostomy. The catheter of the pump was then introduced into the basal turn of the cochlea into the perilymphatic space (scala tympani) via the cochleostomy (Fig. 7). The body of the pump was inserted in a subcutaneous pocket and the skin incision closed in layers.

Example 4: Assessment of Transformation of Cochlear Cells by Administration of Recombinant AAV

AAV transfused cochleae were assayed for expression of the marker gene, β-galactosidase, via *in situ* immunohistochemistry. Two weeks post-cochleostomy, animals were sacrificed by intraperitoneal injection of an overdose of sodium phenobarbital (250 mg/kg) and bilateral thoracotomy. Temporal bones were harvested from both sides of the head. Each bulla was opened using rongeurs to expose the cochlea, the stapes was removed, and the cochlea fixed by perfusion of 4.5% paraformaldehyde through the round window. The cochlea was then removed from the remaining temporal bone and immersed

in 4.5% paraformaldehyde overnight at 4°C. After complete fixation, specimens were decalcified in 0.2 M EDTA/1X PBS/4.5% paraformaldehyde for 2-3 weeks with at least three solution changes. Following decalcification, the specimens were placed in 0.9% saline, dehydrated through a graded alcohol series and then xylenated. Specimens were embedded in paraffin and sectioned at 4-8 μm on a microtome (Leica RM2035).

The paraffin-embedded cochlear sections were dewaxed, blocked with 10% NHS, 0.1% Tween 20 in PBS, and hybridized overnight with mouse anti-*E. coli* β-gal antibody. The sections were washed to remove unbound antibody and then hybridized with a secondary antibody (biotin labeled anti-mouse IgG monoclonal antibody. Bound label was amplified using the ABC reagent (Vector) and developed with DAB. Sections were examined under low and high power magnification and the presence or absence of staining in different parts of the cochlea noted.

Each of the cochleae transfused with recombinant AAV stained positively for β-gal expression throughout its entire length, whereas the saline-infused cochlea were devoid of staining. β-gal expression was detected in nearly all tissue types within the cochlea including the spiral ligament, spiral limbus, and organ of Corti including the auditory hair cells, and the spiral ganglion cells. Particularly intense staining was noted in the perivascular region within the cochlea in several animals. β-gal expression was greater within the spiral ligament and the spiral limbus. The stria vascularis that encases the cochlear duct remained selectively unstained in all AAV infused cochleae. The β-gal expression was the strongest in the basal turn of the cochlea and decreased in the subsequent turns toward the cochlear apex. This tissue and regional selectivity within the cochlea may be consequence of differential susceptibility of inner ear cells/tissue to AAV transfection, and/or the viral titre gradient generated by the mode of vector delivery to the cochlea and its subsequent dissemination in the perilymphatic and endolymphatic space.

Analysis of the contralateral cochlea from an AAV perfused animal revealed weak staining within the same tissues as the perfused cochlea indicating presence of the virus and

expression of the β-gal marker gene. Expression of the virus, distant from the site of the infection may be due to its hematogenous dissemination to near and distant tissues, or migration of AAV via the bone marrow space of the temporal bone or via the cerebrospinal fluid space to the contralateral ear, since the perilymphatic space into which the virus is perfused is directly connected to the CSF via the cochlear aqueduct. Cochlear sections from saline-perfused and unoperated animals did not show evidence of β-gal expression.

The cellular and the tissue architecture within the AAV-perfused cochlea was intact and free from inflammation throughout with the exception of minimal trauma at the site of cochleostomy. There was no evidence of endolymphatic hydrops in any of the animals.

Example 5: Long-Term Transgene Expression within Guinea Pig Cochlea Using A Recombinant AAV Viral Vector

As described above, recombinant AAV viral vector particles were prepared using the β-gal-expressing construct shown in Fig. 10. Approximately 10^5 particles of AAV containing the CMV-driven β-gal expression construct were infused into the cochlea of guinea pigs with the aid of a an osmotic minipump as described above in Example 3. Animals were sacrificed after 1, 2, 4, 8, 12, and 24 weeks. Four Hartley guinea pigs infused with saline and four nonimplanted animals served as negative control. The infused cochleae was harvested from each animal, processed as paraffin sections, and assayed for β-gal expression using immunohistochemistry. Animals infused with AAV showed immunoreactivity in the spiral limbus, spiral ligament, spiral ganglion cells, and the organ of Corti. This patter of β-gal expression was observed in all animals from 1 through 24 weeks post-infusion; the relative intensity of immunoreactivity decreased progressively with duration. Cochleae from saline-infused and noninfused animals were devoid of immunoreactivity. Th observed 24 week expression of the β-gal marker gene represents the longest documented expression of a transgene within the inner ear following localized AAV-mediated introduction.

Example 6: Transformation and Expression of Green Fluorescent Protein (GFP) in Guinea Pig Cochlea

Recombinant AAV viral vector particles were prepared using the GFP-expressing construct shown in Fig. 10. Approximately 10^5 particles of AAV containing the CMV-driven GFP expression construct were infused into the cochlea of guinea pigs over two days to one week with the aid of a an osmotic minipump as described above in Example 3. Animals infused with saline and noninfused animals were used as negative controls. Cochleae were fixed, decalcified, and embedded in paraffin. Sections of 8 μm were cut dewaxed, coverslipped, and viewed under fluorescence. Animals infused with AAV containing GFP showed intense fluorescent activity in spiral ganglia, Reissner's membrane, spiral ligament, and organ of Corti as compared to saline and noninfused animals.

Example 7: Gene Therapy of the Human Inner Ear

Recombinant viral vectors and recombinant AAV particles are prepared as described above such that the viral vector expresses a nucleotide sequence encoding a therapeutic gene product of interest. An osmotic minipump is filled with approximately 100 μl to 1000 μl PBS containing the recombinant adeno-associated virus.

Placement of the minipump in the patient can be achieved by a number of different incisions. After the incision is made, the periosteum is elevated off the mastoid. In adults, the temporalis muscle is left *in situ* at this stage, whereas in children it is elevated with the flap. After performing an intact canal wall mastoidectomy, the facial recess is opened. A cochleostomy is then created by drilling anteriorly from the round window into the basal turn of the cochlea. The catheter of the osmotic minipump is introduced into the basal turn of the cochlea into the perilymphatic space (scala tympani) via the cochleostomy. The osmotic minipump is filled with a transforming vector solution composed of recombinant AAV viral particles encoding a nucleotide sequence of interest (e.g., a nerve growth factor (e.g., NGF (Fig. 9), NT-3 (Fig. 7), BDNF (Fig. 8) suspended in saline. The solution in the mini-pump can additionally

contain other agents desirable for introduction into the inner ear (e.g., antiinflammatory agents). The body of the pump is inserted in a subcutaneous pocket, the round window and facial recess packed with gelfoam pledgets, and the skin incisions closed with sutures. The osmotic minipump is left in place for several days to several weeks to allow infusion of the recombinant viral particles into the inner ear and transformation of inner ear cells, and may be left in place for as long as desired.

Example 8: Gene Therapy of the Human Inner Ear in Conjunction with Cochlear Implant

The method of the invention can be performed in conjunction with cochlear implantation. Cochlear implantation can be achieved by a number of different incisions for the placement of the cochlear implant device. It is important to design a flap that is both well-vascularized and large enough to accommodate the receiver. In addition, it is desirable that both the device and its lead wires be located well away from the skin incision. After the incisions is made, the periosteum is elevated off the mastoid. In adults, the temporalis muscle is left *in situ* at this stage, whereas in children it is elevated with the flap. A dummy device is used as a template to mark the location for the receiver, and a well is drilled into the calvarium to seat the receiver. A trough is drilled to accommodate the lead wire in its route to the mastoid cavity. Drill holes are placed on either side of the well to serve as anchors for retention sutures.

After performing an intact canal wall mastoidectomy, the facial recess is opened. A cochleostomy is the created by drilling anteriorly from the round window into the basal turn of the cochlea. In the Clarion device (Minimed, Inc., Sylmar, CA) an electrode inserter is utilized to facilitate placement of the active lead of the receiver into the scala tympani. This tool straightens the coiled electrode to ease its insertion. After securing the receiver in the bony well with sutures, the multichannel electrode is gently eased into the scala tympani. The lead wire is secured by a suture through the mastoid cortex and then coiled in the mastoid cavity.

In addition, the catheter of the osmotic minipump is also introduced into the basal turn of the cochlea into the perilymphatic space (scala tympani) via the cochleostomy. The osmotic minipump is filled with a transforming vector solution composed of recombinant AAV viral particles encoding a nucleotide sequence of interest (e.g., a nerve growth factor (e.g., NGF (Fig. 9), NT-3 (Fig. 7), BDNF (Fig. 8) suspended in saline. The solution in the minipump can additionally containing other agents desirable for introduction into the inner ear (e.g., antiinflammatory agents). The body of the pump is inserted in a subcutaneous pocket, the round window and facial recess packed with gelfoam pledgets, and the skin incisions closed with sutures. The osmotic minipump is left in place for as long as desired (e.g., for several days to several weeks to several years) to allow infusion of the recombinant viral particles into the inner ear and transformation of inner ear cells.

Example 9: Transformation of Rat Cochlear Cells *In Vitro*

Recombinant viral vectors and recombinant AAV particles containing β-gal-encoding DNA were prepared as described above using the CMV-driven β-gal expression construct (Fig. 10). Dissection of cochlear explants and spiral ligament/stria vascularis explant followed the methods of Zheng et al. (1995, *supra*; 1996, *supra*). The cochleae were dissected from postnatal day (PN) 3 Wistar rats. After spiral ligament and stria vascularis tissues were removed and kept, the remaining cochlear explant containing the spiral ganglion and organ of Corti was cut into three pieces consisting of basal, middle, and apical turns.

Infection of cochlear explants was carried out in a nonsilated eppendorf tube containing 10-20 μl of serum-free media with approximately 10^6 viral particles/ml. The explants are then incubated at 37°C for three hours. Experiments have shown that prolonged incubation in the infection module (>12 hrs) decreases the viability of organotypic culture and disturbs the normal architecture. After the infection, the explants were maintained as organotypic cultures using a modification of the explant culturing system of Zheng et al.

(Zheng et al. 1995 J. Neurosci. 15(7):5079-5087; Zheng et al. 1996 Eur. J. Neurosci. 8:1897-1905). Briefly, Lab-Tek 8 well culture wells were pre-coated with rat tail collagen (type I, 4.5 mg/ml) diluted 1:50 and formulated in 0.02 N acetic acid in water. Approximately 200 µl was used to coat the wells, which were then incubated at 37°C for 1-2 hrs, and washed twice with water prior to plating with explants. The cochlear explants were gently laid down on the collagen-coated culture wells with 200 µl of serum-free medium [BME + serum-free supplement (insulin, transferrin, sodium selenite, Sigma I-1884), 1% BSA, 2 mM glutamine, 5 mg/ml glucose] without antibiotics. The culture media was changed every two days. Uninfected cochlear explants (mock infected) served as a control.

After 72 hours of incubation, the cochlear explants were assayed for β-gal expression by histochemical detection using the methods described by Sanes et al. (1986 EMBO J. 5:3133-3142) with modifications. Briefly, the explants were fixed for 15 min at 4°C in 2% formaldehyde and 0.2% glutaraldehyde in PBS, and then washed three times with PBS + 1mM $MgCl_2$. The explants were immersed in histochemical mixture of 1 mg/ml X-gal, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 2 mM $MgCl_2$, 0.02% NP-40, and 0.01% sodium deoxycholate in PBS. Incubation was done at 37°C for 6 to 10 hrs.

No observable toxic effects, hair cell degeneration, or degeneration of various cochlear cell types occurred under either the culture conditions alone (control samples) or following AAV infection. Fig. 13, bottom left panel shows an exemplary transformed organotypic rat cochlear explant after infection with AAV-β-gal and X-gal staining. Several transformed cells expressing β-gal are observable within the cochlear structure; a slight endogenous β-gal activity is detectable in the vicinity of the outer hair cells. At higher magnification (Fig. 13, right panel) shows a high magnification of the cochlear explant in the region of outer hair cells and Hansen's cells. Numerous Hansen's cells expressing β-gal following AAV-β-gal infection are noted.

In order to more closely examine the β-gal expression of cochlear cells, dissociated cultures of spiral ganglion neurons (SGN) were prepared. SGN cell cultures were prepared according to methods well known in the art. Briefly, after dissection of the cochlea and removal of spiral ligament and stria vascularis tissues, the remaining spiral ganglion cells were incubated in a mixture of 0.125% trypsin and 0.125% collagenase for 25-30 min at 37°C. The enzyme was inactivated with a mixture of 0.005% soybean trypsin inhibitor and 0.005% DNASE before trituration with 0.05% DNase in BME. The dissociated cells were counted on hemocytometer and plated at a density of ~80,000/well on polylysine (100 μg/ml)/ laminin (10 μg/ml) coated 16-well Lab-Tek slides with 200 μl of serum free media as described above. Infection of dissociated SGN was accomplished by introducing approximately 10^6 AAV-β-gal viral particles into the media. The infection was allowed to continue for 6 hrs at which time media was replaced with fresh media.

Fig. 13, top left panel shows a culture of dissociated rat cochlear cells infected with AAV-β-gal. As shown, a substantial number of cochlear cells in the test samples were successfully transformed with the β-gal-encoding construct and expressed β-gal at readily detectable levels. Uninfected dissociated cochlear cultures demonstrated no blue cells.

Example 10: Prevention of Amikacin-Induced Auditory Nerve Damage in BDNF-Transgenic Cochlea

Recombinant viral vectors and recombinant AAV particles containing brain-derived neurotrophin factor-encoding DNA (see the construct in Fig. 8 were prepared as described above. Cochlear explants were prepared as described above and infected with approximately 10^6 particles/ml of AAV containing the BDNF expression construct according to the protocols described above. Uninfected cochlear explants (mock infected) served as a control. The cochlear explants were then maintained in organotypic culture as described above.

Three days after infection, the ototoxic drug amikacin was introduced into the culture media of the control cochlear (mock-infected) and AAV-BDNF transfected cochlear explants to

a concnetration of 4.5 mM. After incubation for 2 days, the cochleae's neurofilaments were examined via *in situ* fluorescent immunohistochemistry using AN anti-neurofilament antibody. The samples were first fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 30 min. The fixed cultures were then washed with PBS, and then incubated with the primary anti-neurofilament antibody (N52, Boehringer) in 1% Triton X-100 containing 3% normal goat serum at 4°C for approximately 48 hrs. After washing in PBS, the samples were then incubated at room temperature for 1 hr with species-specific secondary antibody conjugated to Texas Red. The samples were then washed with PBS, mounted in Fluoromount-G (Southern Biotech. Associates) and visualized under fluorescent microscope.

As shown in Fig. 14, top left panel, numerous radial neurite projections are observed in the control cochlea (mock infected, no amikacin). However, the number of neurite projections in amikacin-exposed control cochlea (mock-infected plus amikacin) was dramatically reduced (Fig. 14, top right panel). In contrast, AAV-BDNF infected explants exposed to amikacin exhibited a significantly increased number of peripheral neurites compared to the uninfected amikacin-exposed explant (Fig. 14, bottom left and right panels). These data demonstrate not only that the cochlear cells were successfully transformed and expressed BDNF, but also that BDNF expression acts as a potent survival factor for SGN exposed to an ototoxic drug.

Following procedures similar to those described above, other therapeutic proteins can be expressed from DNA inserted in the genome of an inner ear cell by gene transfer according to the invention.

The invention now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the appended claims.

What is claimed is:

1. A method of transforming *in vivo* a cell of an inner ear with a nucleotide sequence of interest, comprising:

introducing into an inner ear a vector comprising a construct, the construct comprising a nucleotide sequence of interest and a eukaryotic promoting sequence operably linked to the nucleotide sequence of interest.

2. The method of claim 1, wherein the cell of the inner ear is a cochlear cell.

3. The method of claim 2, wherein the cochlear cell is cell of a cochlear structure selected from the group consisting of the spiral ligament, spiral limbus, stria vascularis, organ of Corti, and Reissner's membrane.

4. The method of claim 2, wherein the cochlear cell is an auditory hair cell, a spiral ganglion cell or a Hansen's cell.

5. The method of claim 1, wherein said introducing is by infusion.

6. The method of claim 1, wherein the viral vector is an adeno-associated viral vector.

7. A substantially enriched genetically transformed cochlear cell culture, comprising:

a nucleotide sequence of interest which expresses a therapeutically effective gene product which nucleotide sequence is artificially and operatively inserted in the genome of the cell;

a promoter operatively linked to the nucleotide sequence.

8. A method of treatment, comprising:

genetically transforming *in vivo* a cell of an inner ear of a subject with a vector comprising a construct, the construct comprising a DNA of interest which expresses a gene product that the patient is in need of and a eukaryotic promoting sequence operably linked to the DNA of interest; and

allowing the genetically transformed cells to express the gene product in a therapeutically effective amount thereby treating the patient.

9. The method of claim 8, wherein the cell of the inner ear is a cochlear cell.

10. The method of claim 9, wherein the cochlear cell is an auditory hair cell, a spiral ganglion cell or a Hansen's cell.

11. The method of claim 8, wherein said introducing is by infusion.

12. The method of claim 8, wherein the nucleotide sequence of interest encodes a nerve growth factor.

13. The method of claim 8, further comprising implanting a cochlear implant in the inner ear of the subject.

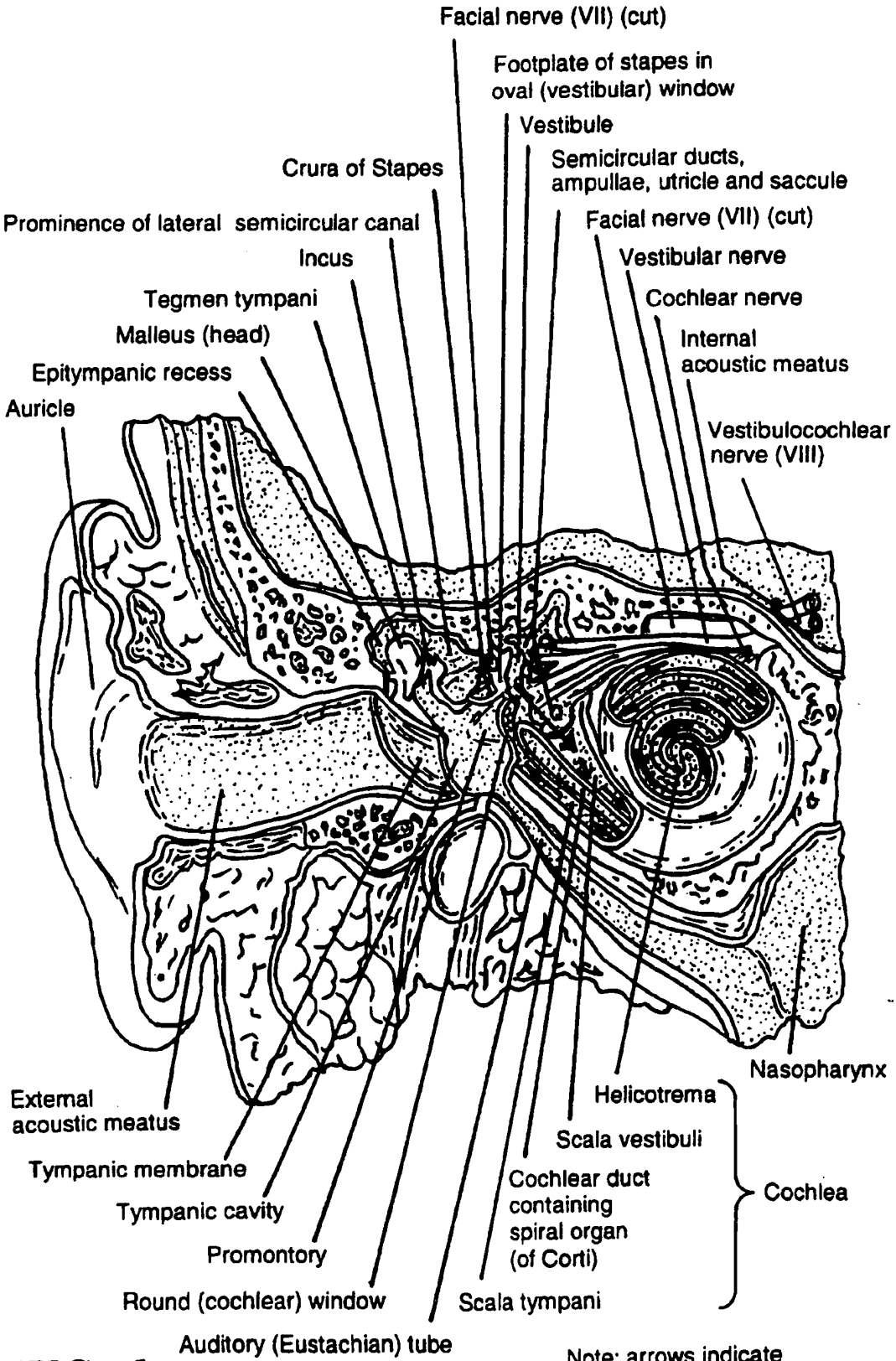
Facial nerve (VII) (cut)
Footplate of stapes in oval (vestibular) window
Vestibule
Semicircular ducts, ampullae, utricle and saccule
Crura of Stapes
Facial nerve (VII) (cut)
Prominence of lateral semicircular canal
Vestibular nerve
Incus
Cochlear nerve
Tegmen tympani
Internal acoustic meatus
Malleus (head)
Epitympanic recess
Auricle
Vestibulocochlear nerve (VIII)
Nasopharynx
Helicotrema
External acoustic meatus
Scala vestibuli
Tympanic membrane
Cochlear duct containing spiral organ (of Corti)
Cochlea
Tympanic cavity
Promontory
Round (cochlear) window
Scala tympani
Auditory (Eustachian) tube
Note: arrows indicate course of sound waves

FIG. 1

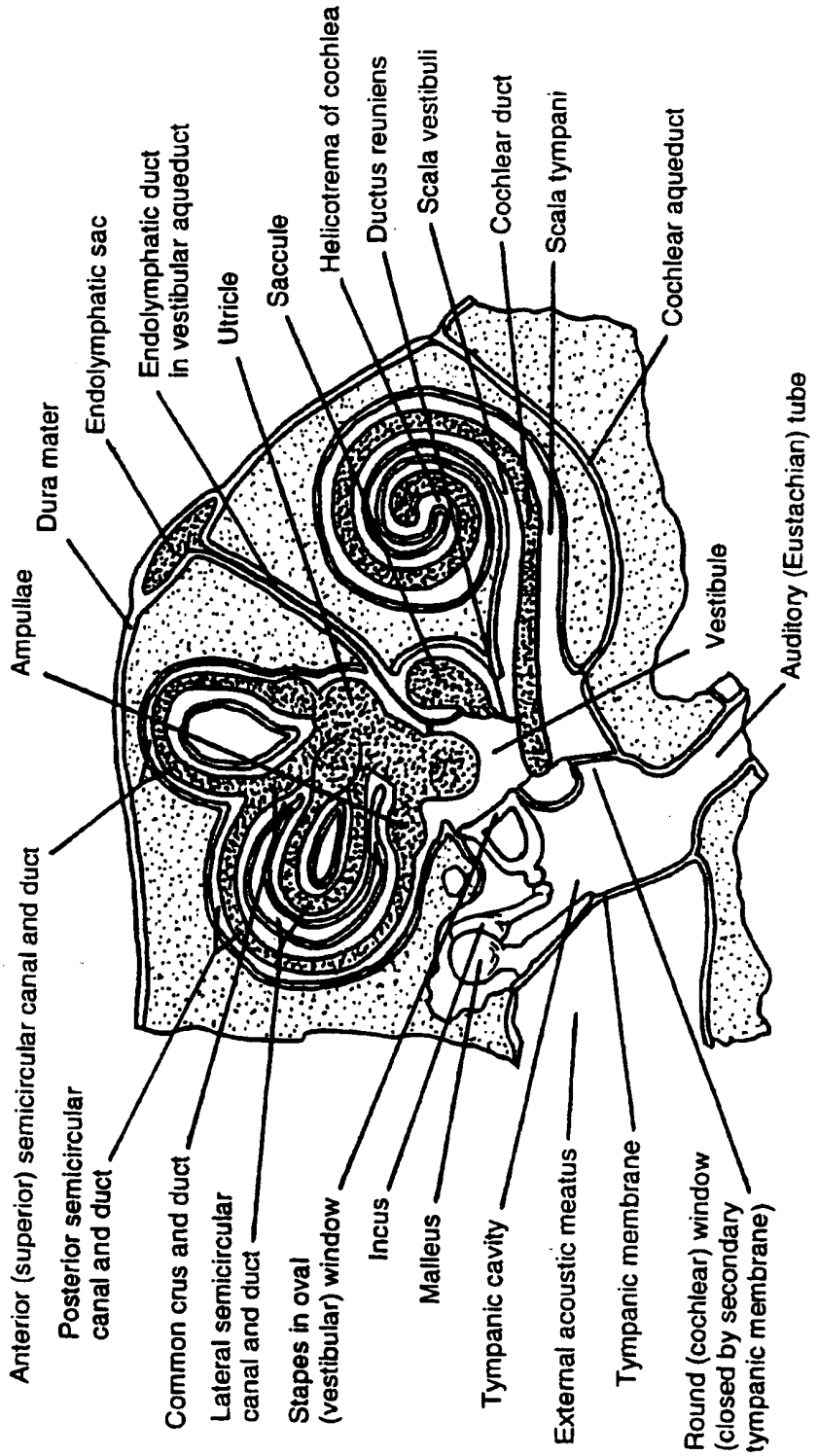
Anterior (superior) semicircular canal and duct
Posterior semicircular canal and duct
Common crus and duct
Lateral semicircular canal and duct
Stapes in oval (vestibular) window
Incus
Malleus
Tympanic cavity
External acoustic meatus
Tympanic membrane
Round (cochlear) window (closed by secondary tympanic membrane)
Ampullae
Dura mater
Endolymphatic sac
Endolymphatic duct in vestibular aqueduct
Utricle
Saccule
Helicotrema of cochlea
Ductus reuniens
Scala vestibuli
Cochlear duct
Scala tympani
Cochlear aqueduct
Vestibule
Auditory (Eustachian) tube

FIG. 2

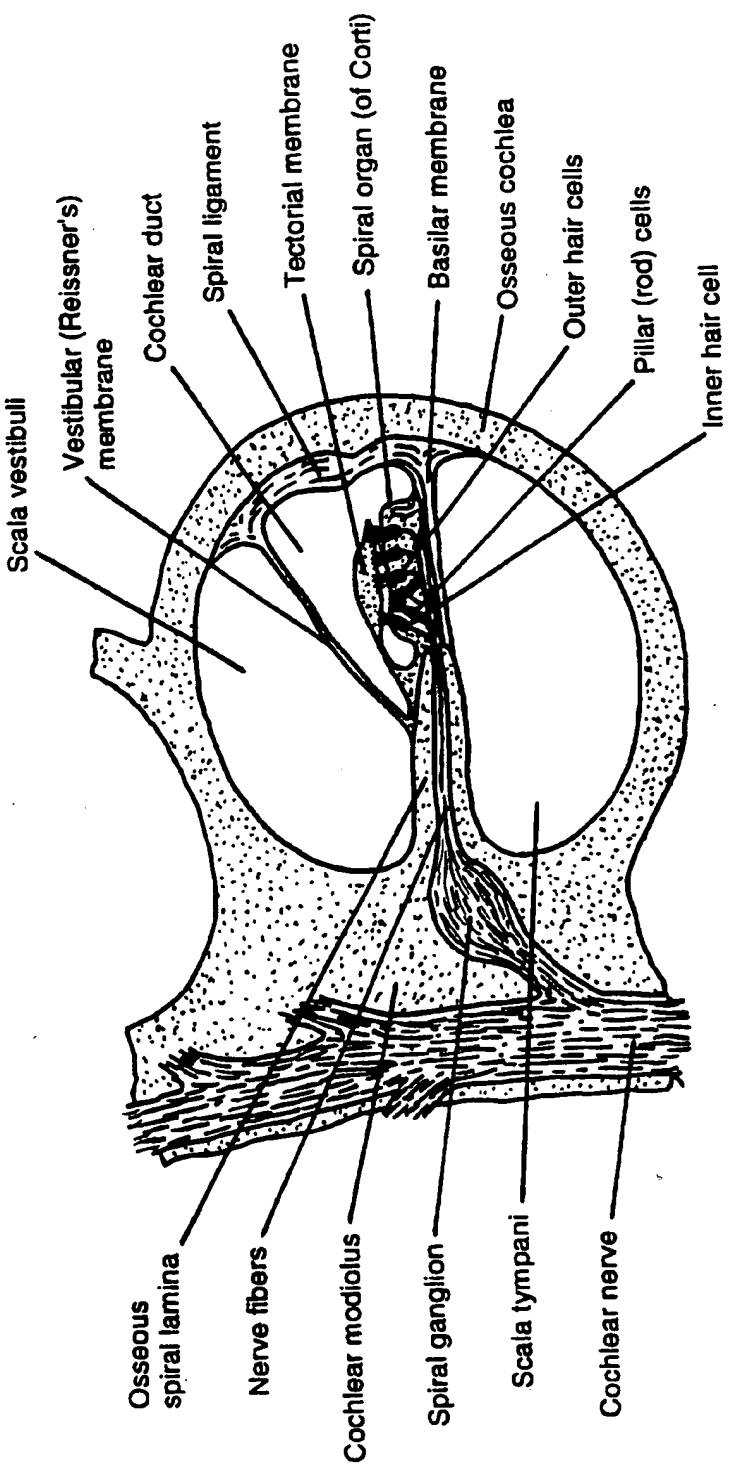
Scala vestibuli
Vestibular (Reissner's) membrane
Cochlear duct
Spiral ligament
Tectorial membrane
Spiral organ (of Corti)
Basilar membrane
Osseous cochlea
Outer hair cells
Pillar (rod) cells
Inner hair cell
Osseous spiral lamina
Nerve fibers
Cochlear modiolus
Spiral ganglion
Scala tympani
Cochlear nerve

FIG. 3

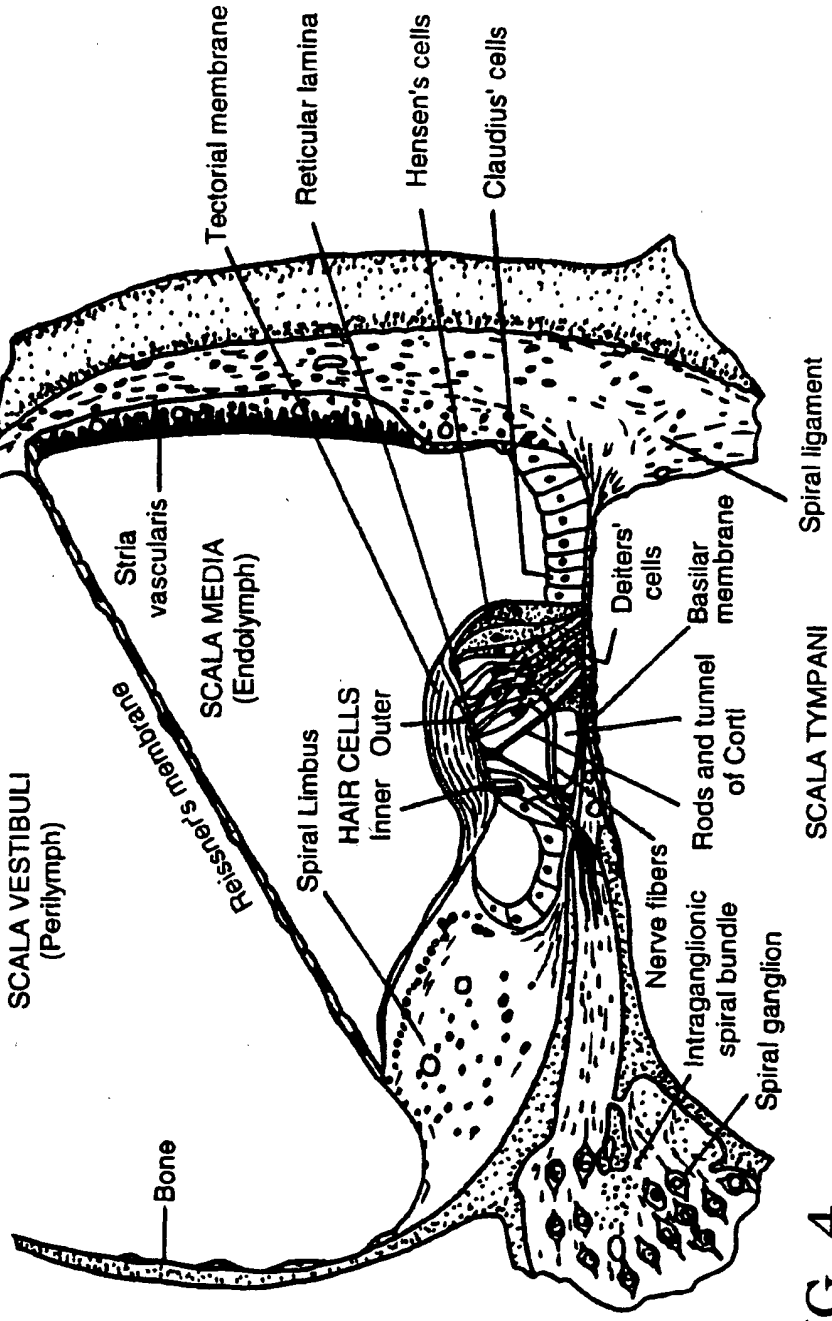
Tectorial membrane
Reticular lamina
Hensen's cells
Claudius' cells
Spiral ligament
Stria vascularis
SCALA MEDIA (Endolymph)
Deiters' cells
Basilar membrane
Reissner's membrane
Spiral Limbus
HAIR CELLS Outer
Inner
Rods and tunnel of Corti
SCALA TYMPANI (Perilymph)
SCALA VESTIBULI (Perilymph)
Nerve fibers
Intraganglionic spiral bundle
Spiral ganglion
Bone

FIG. 4

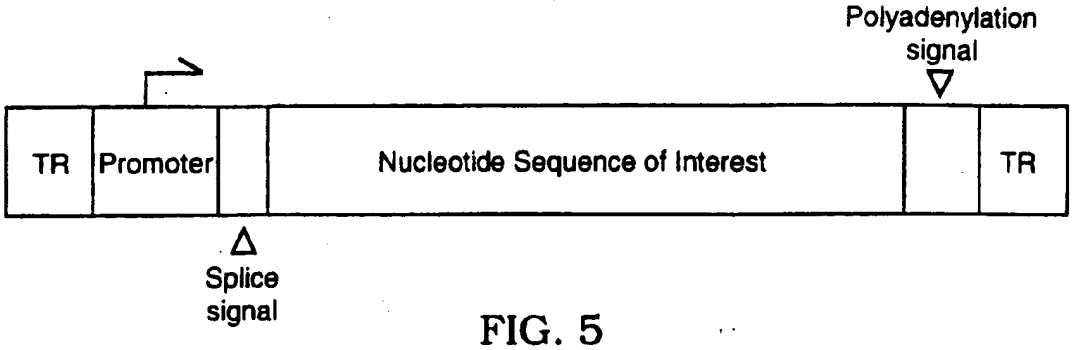

FIG. 5

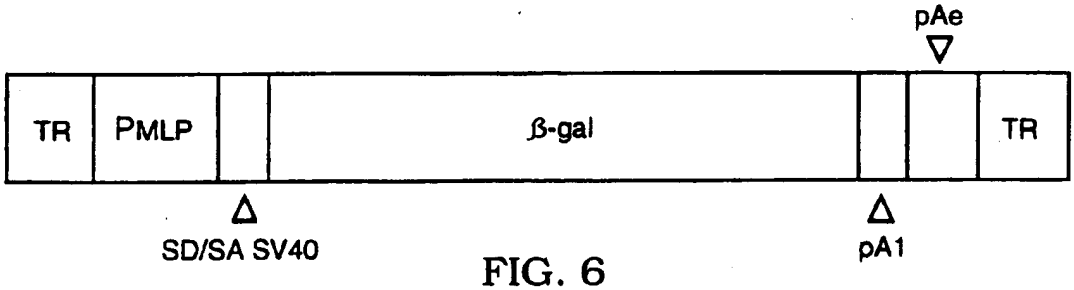

FIG. 6

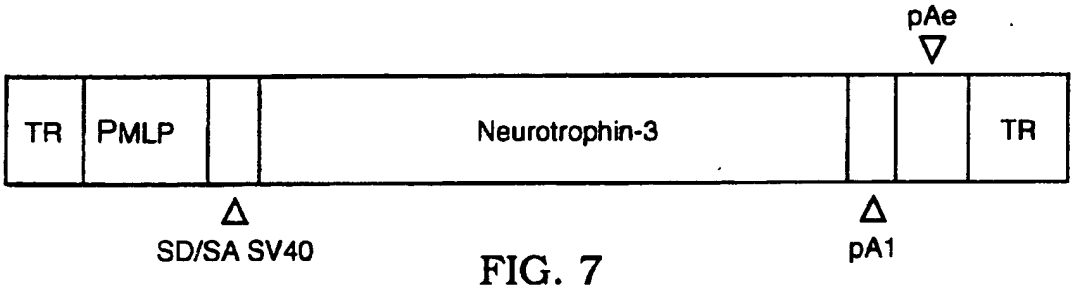

FIG. 7

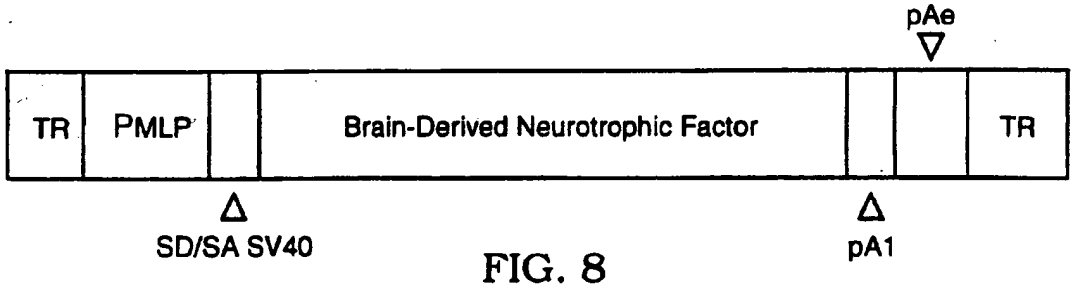

FIG. 8

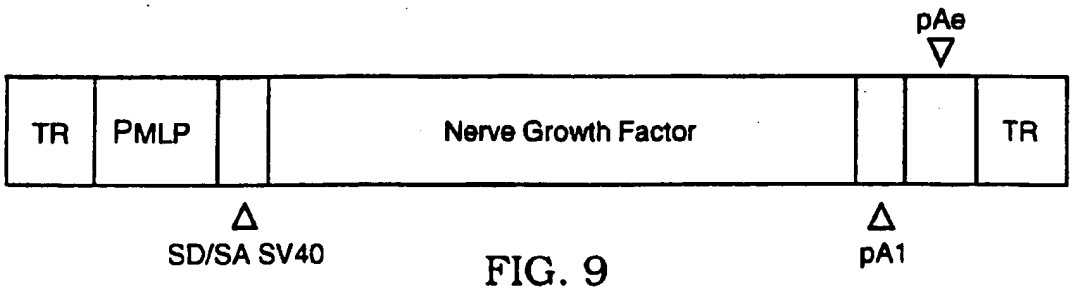

FIG. 9

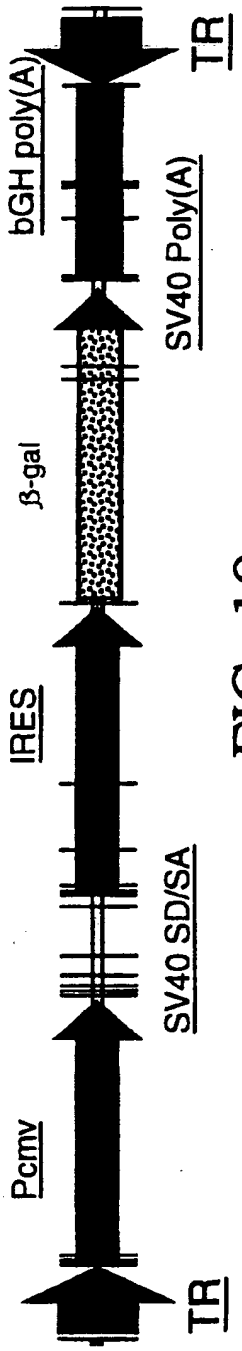
TR
bGH poly(A)
SV40 Poly(A)
β-gal
IRES
SV40 SD/SA
Pcmv
TR

FIG. 10

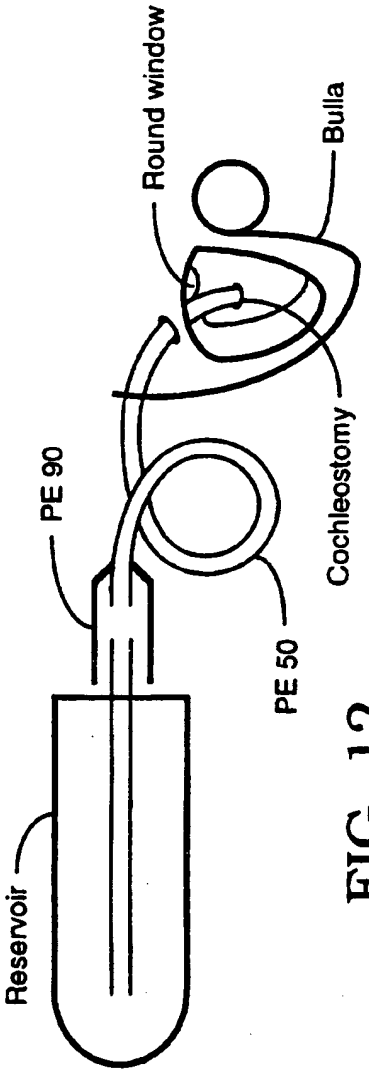
Round window
Bulla
PE 90
Cochleostomy
PE 50
Reservoir

FIG. 12

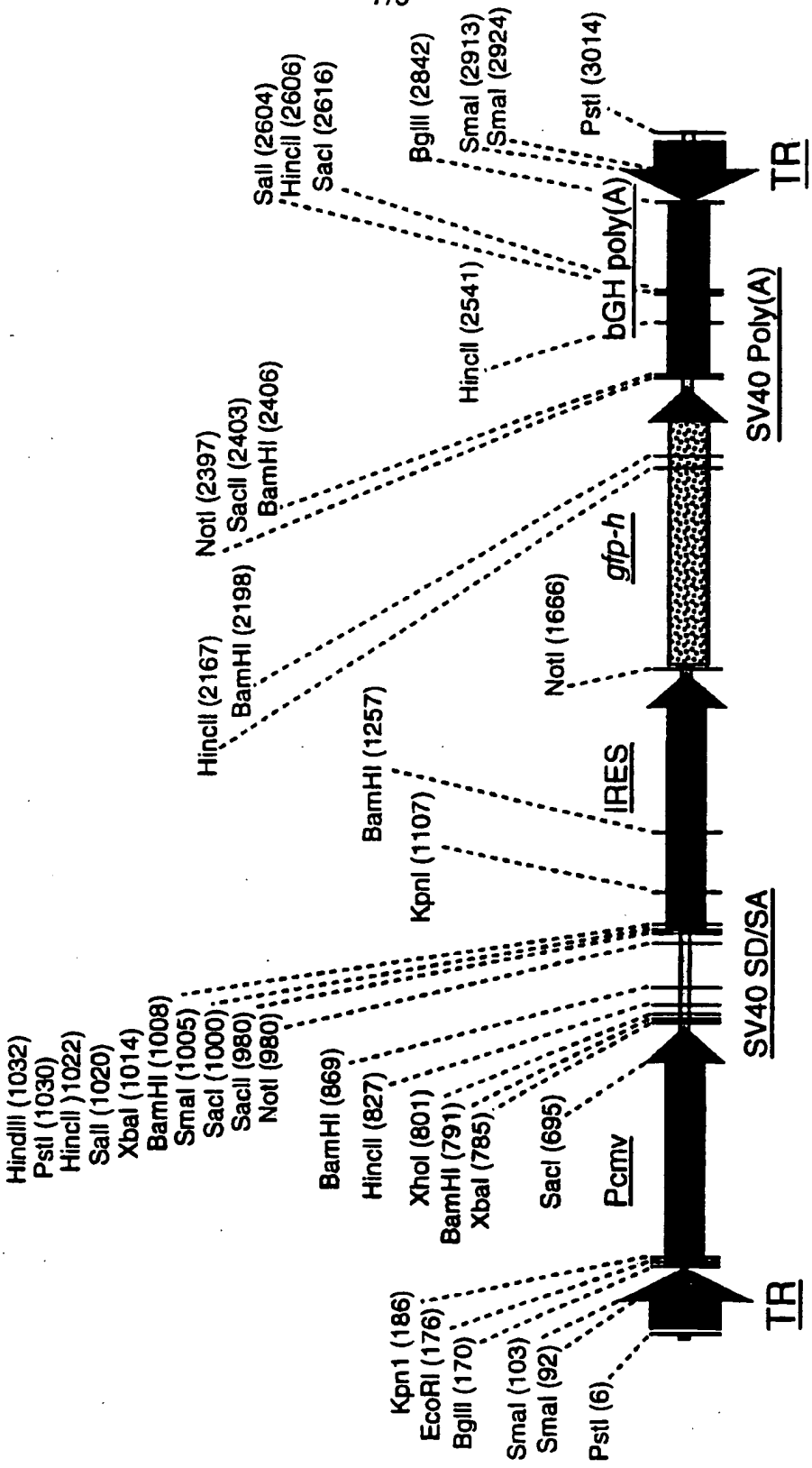

FIG. 11

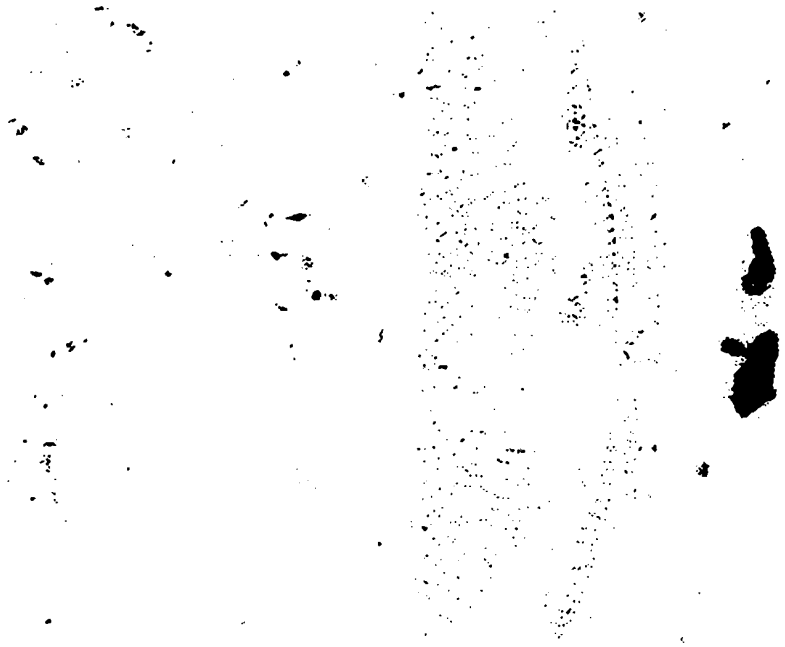

FIG.13

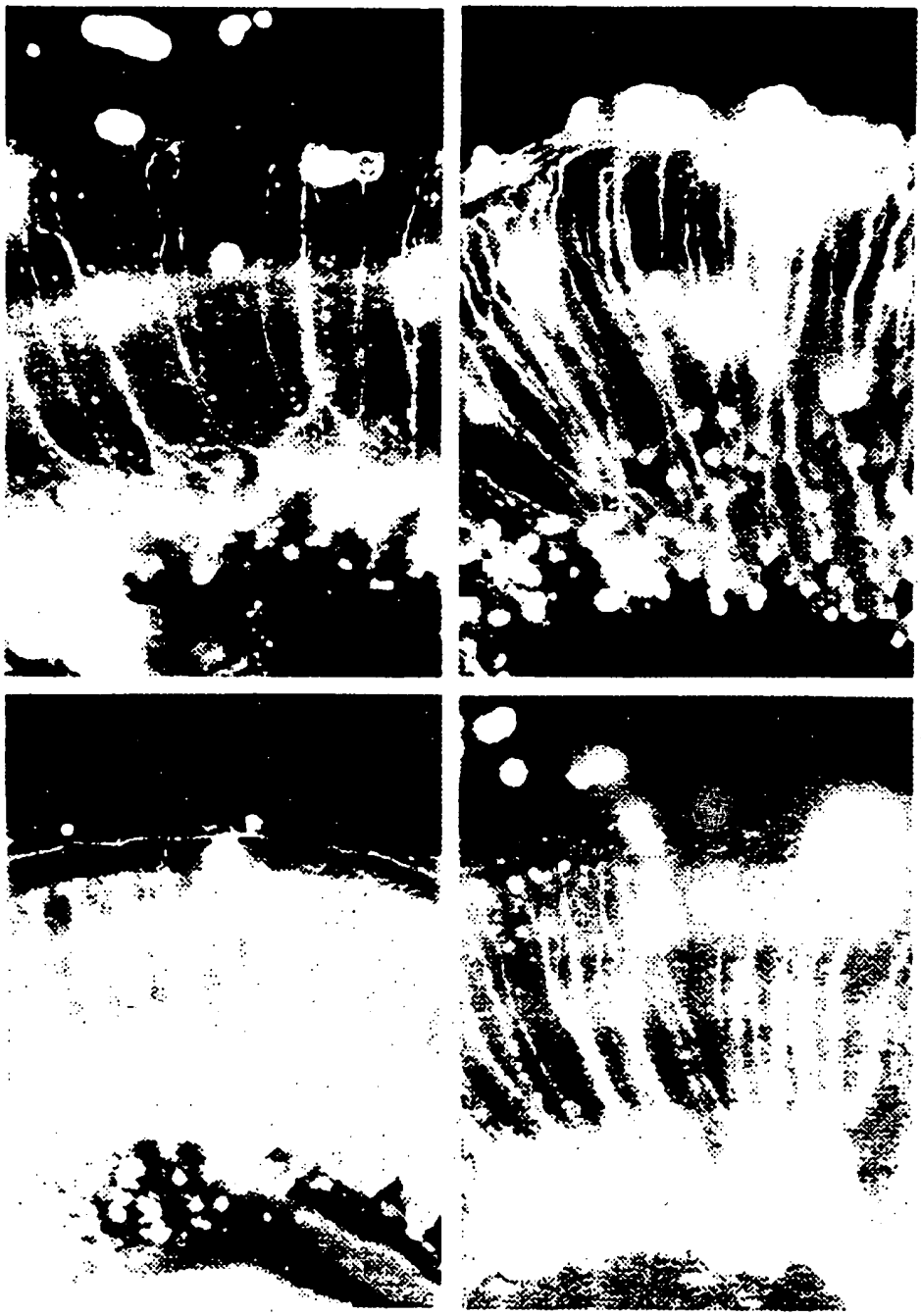

FIG.14

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/11602

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A01N 43/04; A61K 31/70; C12N 15/00, 15/63, 15/70
US CL :514/44; 435/320.1, 172.3; 935/22, 33, 34
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/44; 435/320.1, 172.3; 935/22, 33, 34

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DIALOG: BIOSIS, MEDLINE, EMBASE, DERWENT; APS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	FLOTTE et al. Stable *in vivo* expression of the cystic fibrosis transmembrane conductance regulator with an adeno-associated virus vector.Proc. Natl. Acad. Sci. USA. November 1993, Vol. 90, pages 10613-10617, see entire document.	1-13
Y	SHAH et al. An extended study: Protective effects of nerve growth factor in neomycin-induced auditory neural degeneration. Am. J. Otology. May 1995, Vol. 16, No. 3, pages 310-314, see entire document.	1-13

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 14 AUGUST 1997	Date of mailing of the international search report 05 SEP 1997
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer ABDUR RAZZAQUE Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*